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<b>(54) Title:</b> A PROCESS OF DELIVERING A POLYNUCLEOTIDE VIA THE VASCULAR SYSTEM  <b>(57) Abstract</b>  A process for delivering a polypeptide into a parenchymal cell in a mammal, comprising, transporting the polynucleotide into a blood vessel communicating with the parenchymal cell in tissue or organ of the mammal such that the polynucleotide is transfected into the parenchymal cell.		

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## A Process of Delivering a Polynucleotide Via the Vascular System

### Field of the Invention

The invention generally relates to techniques for transferring genes into mammalian parenchymal cells *in vivo*. More particularly, a method is provided for  
5   transfecting parenchymal cells with polynucleotides delivered intravascularly.

### Background of the Invention

It was first observed that the *in vivo* injection of plasmid DNA into muscle enabled the expression of foreign genes in the muscle (Wolff, J A, Malone, R W, Williams, P, et al. Direct gene transfer into mouse muscle *in vivo*. *Science*  
10   1990;247:1465-1468.). Since that report, several other studies have reported the ability for foreign gene expression following the direct injection of DNA into the parenchyma of other tissues. Naked DNA was expressed following its injection into cardiac muscle (Acsadi, G., Jiao, S., Jani, A., Duke, D., Williams, P., Chong, W., Wolff, J.A. Direct gene transfer and expression into rat heart *in vivo*. The  
15   New Biologist 3(1), 71-81, 1991.), pig epidermis (Hengge, U. R., Chan, E. F., Foster, R. A., Walker, P. S., and Vogel, J. C. *Nature Genetics* 10:161-166 (1995)), rabbit thyroid (M. Sikes, B. O'Malley, M. Finegold, and F. Ledley, *Hum. Gene Ther.* 5, 837 (1994), lung by intratracheal injection (K. B. Meyer, M. M. Thompson, M. Y. Levy, L. G. Barron, F. C. Szoka, *Gene Ther.* 2, 450 (1995)), into arteries using a  
20   hydrogel-coated angioplasty balloon (R. Riessen et al, *Human Gene Ther.* 4, 749 (1993)) (G. Chapman et al. *Circ. Res.* 71, 27 (1992)), melanoma tumors (R. G. Vile and I. R. Hart, *Cancer Res.* 53, 962 (1993)) and rat liver [(Malone, R. W. et al. *JBC* 269:29903-29907 (1994)) (Hickman, M. A. *Human Gene Therapy* 5:1477-1483 (1994))].

25       Another important target tissue for gene therapy is the mammalian liver, given its central role in metabolism and the production of serum proteins. A variety of techniques have been developed to transfer genes into the liver. Cultured hepatocytes have been genetically modified by retroviral vectors [(Wolff, J. A. et al. *PNAS* 84:3344-3348 (1987) (Ledley, F. D., Darlington, G. J., Hahn, T. and Woo,

S.C.L. PNAS 84:5335-5339 (1987)] and re-implanted back into the livers in animals and in people [(J. R. Chowdhury et al. Science 254, 1802 (1991) (M. Grossman et al. Nature Genetics 6, 335 (1994)]. Retroviral vectors have also been delivered directly to livers in which hepatocyte division was induced by partial  
5 hepatectomy [(Kay, M.A. et al Hum Gene Ther. 3:641-647 (1992) (Ferry, N., Duplessis, O., Houssin, D., Danos, O. and Heard, J.-M. PNAS 88:8377-8381 (1991) (Kaleko, M., Garcia, J.V. and Miller, A.D. Hum Gene Ther. 2:27-32 (1991)]. The injection of adenoviral vectors into the portal or systemic circulatory systems leads to high levels of foreign gene expression that is transient [(L. D.  
10 Stratford-Perricaudet, M. Levrero, J. F. Chasse, M. Perricaudet, P. Briand, Hum. Gene Ther. 1, 241 (1990) (H. A. Jaffe et al. Nat. Genet. 1, 372 (1992) (Q. Li, M. A. Kay, M. Finegold, L. D. Stratford-Perricaudet, S. L. C. Woo, Hum. Gene Ther. 4, 403 (1993)]. Non-viral transfer methods have included polylysine complexes of asialoglycoproteins that are injected into the system circulation [Wu, G. Y. and  
15 Wu, C. H. J. Biol. Chem. 263:14621-14624 (1988)].

Foreign gene expression has also been achieved by repetitively injecting naked DNA in isotonic solutions into the liver parenchyma of animals treated with dexamethasone [(Malone, R. W. et al. JBC 269:29903-29907 (1994) (Hickman, M. A. Human Gene Therapy 5:1477-1483 (1994)]. Plasmid DNA expression in the  
20 liver has also been achieved via liposomes delivered by tail vein or intraportal routes [(Kaneda, Y., Kunimitsu, I. and Uchida, T. J. Biol. Chem. 264:12126-12129 (1989) (Soriano, P. et al. PNAS 80:7128-7131 (1983) Kaneda, Y., Iwai, K. and Uchida, T. Science 243:375-378 (1989)].

Despite this progress, there is still a need for a gene transfer method that can  
25 efficiently and safely cause the expression of foreign genes in the liver in a and/or repetitive manner.

#### Summary of The Invention

The present invention provides for the transfer of polynucleotides into parenchymal cells within tissues in situ and in vivo. An intravascular route of  
30 administration enables a prepared polynucleotide to be delivered to the parenchymal cells more evenly distributed and more efficiently expressed than

direct parenchymal injections. The efficiency of polynucleotide delivery and expression was increased substantially by increasing the permeability of the tissue's blood vessel. This was done by increasing the intravascular hydrostatic (physical) pressure and/or increasing the osmotic pressure. Expression of a foreign DNA was  
5 obtained in mammalian liver by intraportally injecting plasmid DNA in a hypertonic solution and transiently clamping the hepatic vein/inferior vena cava. Optimal expression was obtained by clamping the portal vein and injecting the hepatic vein/inferior vena cava.

A process is described for delivering a polypeptide into a parenchymal cell  
10 in a mammal, comprising, transporting the polynucleotide into a vessel communicating with the parenchymal cell of the mammal such that the polynucleotide is transfected into the parenchymal cell.

A process for delivering a coded polynucleotide into a parenchymal cell of a mammal for expression of a protein, comprising, transporting the polynucleotide to  
15 a vessel containing a fluid and having a permeable wall; and, increasing the permeability of the wall for a time sufficient to complete delivery of the polynucleotide.

### Brief Description of the Drawings

**Fig. 1(a), (b):** Comparison of total luciferase expression in mice injected with pCILUC under various conditions. The condition abbreviations signify the following: PV-CL, portal vein injections without clamping; PV+CL, portal vein injections with clamping the hepatic vein; IVC-CL, IVC injections without clamping; IVC+CL, IVC injections with clamping the portal vein and hepatic artery; BD-CL, bile duct injections without clamping; BD+CL, bile duct injections with clamping the hepatic vein. Numbers above the bars indicate the number of mice. T bars indicate the standard error.

**Fig. 2:** Scatterplot of the log(e) of luciferase (ng protein) versus peak, intrahepatic, parenchymal pressure (mm Hg). The solid line indicates the best fit. Dotted lines indicate 95% prediction intervals.

**Fig. 3:** Histochemical analysis of  $\beta$ -galactosidase expression in livers after injection of pCILacZ into: A) mouse bile duct with the hepatic vein clamped, B) mouse IVC with the portal vein clamped and C) rat portal vein with the IVC clamped. Mouse injections were done using 100  $\mu$ g of pCILacZ in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution while the rat injections were done using 750  $\mu$ g of pCILacZ in 15 ml of the same solution. Panels A and B were magnified 160X and panel C was magnified 100X.

**Fig. 4:** Human growth hormone levels (Hgh) following the repetitive administration of 100 $\mu$ g of pCMVhGH into the bile duct of mice via a cannula. Numbers identify individual mice.

**Fig. 5(a), (b), (c):** Effects of injection solution volume (a), injection time (b) and preinjection ischemia time (c) on the mean levels of luciferase expression in rat hindlimb muscles. Two days after 475  $\mu$ g of pCILux in 9.5 ml of NSS were injected unilaterally into the iliac artery of adult Sprague-Dawley rats, luciferase activities in 6 hindlimb muscle groups (anterior upper leg, medial upper leg, posterior upper leg, anterior lower leg, posterior lower leg and foot) were measured as previously reported and presented as the means of the total luciferase levels of all 6 muscle groups. The pre-injection ischemia time was 10 min in "a" and "b".

Numbers adjacent to data points indicate the number of animals (1 limb/animal) assayed for each condition. Error bars indicate the standard error.

**Fig. 6:** Effects of various conditions on luciferase expression in rat hind limbs muscles following the injection of pCILux DNA into the iliac artery. Condition 1, 475  $\mu$ g DNA in 9.5 ml of NSS were injected within 10 sec after a 10 min occlusion of limb blood flow; Condition 2, 475  $\mu$ g DNA in 9.5 ml of H<sub>2</sub>O were injected within 10 sec after a 10 min occlusion of limb blood flow; Condition 3, 475  $\mu$ g DNA in 9.5 ml of NSS with 15% mannitol were within 30 sec after a 10 min occlusion of limb blood flow; Condition 4, 80  $\mu$ g of collagenase in 1 ml of NSS were injection into iliac artery immediately after blood flow occlusion. After 10

min, blood flow was unclosed for several sec, closed again, and 475 ug DNA in 9.5 ml of NSS were injected within 10 sec. Bold numbers indicate the number of animals assayed at each experimental conditions. Error bars indicate the standard error.

5

## Detailed Description

### A. Definitions

The term, naked polynucleotides, indicates that the polynucleotides are not associated with a transfection reagent or other delivery vehicle that is required for the polynucleotide to be delivered to the parenchymal cell. A transfection reagent is a compound or compounds used in the prior art that bind(s) to or complex(es) with polynucleotides and mediates their entry into cells. The transfection reagent also mediates the binding and internalization of polynucleotides into cells. Examples of transfection reagents include cationic liposomes and lipids, calcium phosphate precipitates, and polylysine complexes. Typically, the transfection reagent has a net positive charge that binds to the polynucleotide's negative charge. The transfection reagent mediates binding of polynucleotides to cell via its positive charge (that binds to the cell membrane's negative charge) or via ligands that bind to receptors in the cell. For example, cationic liposomes or polylysine complexes have net positive charges that enable them to bind to DNA. Other vehicles are also used, in the prior art, to transfer genes into cells. These include complexing the polynucleotides on particles that are then accelerated into the cell. This is termed biolistic or gun techniques. Other methods include eletroporation in which a device is used to give an electric charge to cells. The charge increases the permeability of the cell.

The term polynucleotide is a term of art that refers to a string of at least two base-sugar-phosphate combinations. Nucleotides are the monomeric units of nucleic acid polymers. The term includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the form of an oligonucleotide messenger RNA, anti-



sense, plasmid DNA, parts of a plasmid DNA or genetic material derived from a virus. A polynucleotide is distinguished, here, from an oligonucleotide by containing more than 120 monomeric units. Anti-sense is a polynucleotide that interferes with the function of DNA and/or RNA.

5

A polynucleotide can be delivered to a cell in order to produce a cellular change that is therapeutic. The delivery of polynucleotides or other genetic material for therapeutic purposes (the art of improving health in an animal including treatment or prevention of disease) is gene therapy. The polynucleotides are coded  
10 to express a whole or partial protein, or may be anti-sense, and can be delivered either directly to the organism in situ or indirectly by transfer to a cell that is then transplanted into the organism. The protein can be missing or defective in an organism as a result of genetic, inherited or acquired defect in its genome. For example, a polynucleotide may be coded to express the protein dystrophin that is  
15 missing or defective in Duchenne muscular dystrophy. The coded polynucleotide is delivered to a selected group or groups of cells and incorporated into those cell's genome or remain apart from the cell's genome. Subsequently, dystrophin is produced by the formerly deficient cells. Other examples of imperfect protein production that can be treated with gene therapy include the addition of the protein  
20 clotting factors that are missing in the hemophilias and enzymes that are defective in inborn errors of metabolism such as phenylalanine hydroxylase. A delivered polynucleotide can also be therapeutic in acquired disorders such as neurodegenerative disorders, cancer, heart disease, and infections. The polynucleotide has its therapeutic effect by entering the cell. Entry into the cell is  
25 required for the polynucleotide to produce the therapeutic protein, to block the production of a protein, or to decrease the amount of a RNA.

Delivery of a polynucleotide means to transfer a polynucleotide from a container outside a mammal to within the outer cell membrane of a cell in the mammal. The term transfection is used herein, in general, as a substitute for the  
30 term delivery, or, more specifically, the transfer of a polynucleotide from directly outside a cell membrane to within the cell membrane. If the polynucleotide is a

primary RNA transcript that is processed into messenger RNA, a ribosome translates the messenger RNA to produce a protein within the cytoplasm. If the polynucleotide is a DNA, it enters the nucleus where it is transcribed into a messenger RNA that is transported into the cytoplasm where it is translated into a protein. The polynucleotide contains sequences that are required for its transcription and translation. These include promoter and enhancer sequences that are required for initiation. DNA and thus the corresponding messenger RNA (transcribed from the DNA) contains introns that must be spliced, poly A addition sequences, and sequences required for the initiation and termination of its translation into protein. Therefore if a polynucleotide expresses its cognate protein, then it must have entered a cell.

A therapeutic effect of the protein in attenuating or preventing the disease state can be accomplished by the protein either staying within the cell, remaining attached to the cell in the membrane or being secreted and dissociating from the cell where it can enter the general circulation and blood. Secreted proteins that can be therapeutic include hormones, cytokines, growth factors, clotting factors, anti-protease proteins (e.g. alpha-antitrypsin) and other proteins that are present in the blood. Proteins on the membrane can have a therapeutic effect by providing a receptor for the cell to take up a protein or lipoprotein. For example, the low density lipoprotein (LDL) receptor could be expressed in hepatocytes and lower blood cholesterol levels and thereby prevent atherosclerotic lesions that can cause strokes or myocardial infarction. Therapeutic proteins that stay within the cell can be enzymes that clear a circulating toxic metabolite as in phenylketonuria. They can also cause a cancer cell to be less proliferative or cancerous (e.g. less metastatic). A protein within a cell could also interfere with the replication of a virus.

The delivered polynucleotide can stay within the cytoplasm or nucleus apart from the endogenous genetic material. Alternatively, the polynucleotide could recombine (become a part of) the endogenous genetic material. For example, DNA can insert into chromosomal DNA by either homologous or non-homologous recombination.

Parenchymal cells are the distinguishing cells of a gland or organ contained in and supported by the connective tissue framework. The parenchymal cells typically perform a function that is unique to the particular organ. The term "parenchymal" often excludes cells that are common to many organs and tissues  
5 such as fibroblasts and endothelial cells within the blood vessels.

In a liver organ, the parenchymal cells include hepatocytes, Kupffer cells and the epithelial cells that line the biliary tract and bile ductules. The major constituent of the liver parenchyma are polyhedral hepatocytes (also known as hepatic cells) that presents at least one side to an hepatic sinusoid and apposed  
10 sides to a bile canaliculus. Liver cells that are not parenchymal cells include cells within the blood vessels such as the endothelial cells or fibroblast cells.

In striated muscle, the parenchymal cells include myoblasts, satellite cells, myotubules, and myofibers. In cardiac muscle, the parenchymal cells include the myocardium also known as cardiac muscle fibers or cardiac muscle cells and the  
15 cells of the impulse connecting system such as those that constitute the sinoatrial node, atrioventricular node, and atrioventricular bundle.

In a pancreas, the parenchymal cells include cells within the acini such as zymogenic cells, centroacinar cells, and basal or basket cells and cells within the islets of Langerhans such as alpha and beta cells.

20 In spleen, thymus, lymph nodes and bone marrow, the parenchymal cells include reticular cells and blood cells (or precursors to blood cells) such as lymphocytes, monocytes, plasma cells and macrophages.

In the nervous system which includes the central nervous system (the brain and spinal cord) peripheral nerves, and ganglia, the parenchymal cells include  
25 neurons, glial cells, microglial cells, oligodendrocytes, Schwann cells, and epithelial cells of the choroid plexus.

In the kidney, parenchymal cells include cells of collecting tubules and the proximal and distal tubular cells. In the prostate, the parenchyma includes epithelial cells.

30 In glandular tissues and organs, the parenchymal cells include cells that produce hormones. In the parathyroid glands, the parenchymal cells include the

principal cells (chief cells) and oxyphilic cells. In the thyroid gland, the parenchymal cells include follicular epithelial cells and parafollicular cells. In the adrenal glands, the parenchymal cells include the epithelial cells within the adrenal cortex and the polyhedral cells within the adrenal medulla.

- 5           In the parenchyma of the gastrointestinal tract such as the esophagus, stomach, and intestines, the parenchymal cells include epithelial cells, glandular cells, basal, and goblet cells.

          In the parenchyma of lung, the parenchymal cells include the epithelial cells, mucus cells, goblet cells, and alveolar cells.

- 10           In fat tissue, the parenchymal cells include adipose cells or adipocytes. In the skin, the parenchymal cells include the epithelial cells of the epidermis, melanocytes, cells of the sweat glands, and cells of the hair root.

          In cartilage, the parenchyma includes chondrocytes. In bone, the parenchyma includes osteoblasts, osteocytes, and osteoclasts.

- 15           An intravascular route of administration enables a polynucleotide to be delivered to parenchymal cells more evenly distributed and more efficiently expressed than direct parenchymal injections. Intravascular herein means within a hollow tubular structure called a vessel that is connected to a tissue or organ within the body. Within the cavity of the tubular structure, a bodily fluid flows to or from the body part. Examples of bodily  
20   fluid include blood, lymphatic fluid, or bile. Examples of vessels include arteries, arterioles, capillaries, venules, sinusoids, veins, lymphatics, and bile ducts. The intravascular route includes delivery through the blood vessels such as an artery or a vein.

- Polypeptide refers to a linear series of amino acid residues connected to one  
25   another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues.

          Protein refers to a linear series of greater than 50 amino acid residues connected one to another as in a polypeptide.

Afferent blood vessels of organs are defined as vessels which are directed towards the organ or tissue and in which blood flows towards the organ or tissue under normal physiologic conditions. Conversely, the efferent blood vessels of organs are defined as vessels which are directed away from the organ or tissue and in which blood  
5 flows away from the organ or tissue under normal physiologic conditions. In the liver, the hepatic vein is an efferent blood vessel since it normally carries blood away from the liver into the inferior vena cava. Also in the liver, the portal vein and hepatic arteries are afferent blood vessels in relation to the liver since they normally carry blood towards the liver.

10

#### B. Delivery of Polynucleotides

In a preferred embodiment of the present invention, a naked polynucleotide is delivered into a liver blood vessel at distal or proximal points. A liver blood vessel includes the portal venous system which transports blood from the gastrointestinal tract  
15 and other internal organs (e.g. spleen, pancreas and gall bladder) to the liver. Another liver blood vessel is the hepatic vein. The hepatic vein may also be reached via the inferior vena cava or another blood vessel that ultimately connects to the liver. A needle or catheter is used to inject the polynucleotide into the vascular system. The injection can be performed under direct observation following an incision and visualization of the tissues blood  
20 vessels. Alternatively, a catheter can be inserted at a distant site and threaded so that it resides in the vascular system that connects with the target tissue. In another embodiment, the injection could be performed by using a needle that traverses the intact skin and enters a vessel that supplies or drains from the target tissue.

In a preferred embodiment, the liver and portal vein of mice (25 g, 6-week  
25 old ICR mice) are visualized through a ventral midline incision. Anesthesia was obtained from intramuscular injections of 1000 µg of ketamine-HCl (Parke-Davis, Morris Plains, NJ) in 1 ml of normal saline and methoxyflurane (Pitman-Moore, Mundelein, IL, USA) which was administered by inhalation as needed. Plasmid DNA in 1 ml of various solutions containing heparin to prevent clotting was

injected into the portal vein using a needle over approximately 30 sec. At various times after the injection, the animals were sacrificed by cervical dislocation and the livers (average weight of 1.5 g) were divided into six sections composed of two pieces of median lobe, two pieces of left lateral lobe, the right lateral lobe, and the caudal lobe plus a small piece of right lateral lobe. Each of the six sections were placed separately into an homogenizing buffer. The homogenates were centrifuged and the supernatant analyzed for the foreign gene product. If the gene product is secreted then blood is obtained from the retro-orbital venous sinus and the level of the secreted protein is assayed in the blood. For example, the expression of the human growth hormone gene can be detected by measuring the amount of human growth hormone in the mouse serum using a radioimmune assay (RIA) (HGH-TGES 100T kit from Nichols Institute, San Juan Capistrano, CA, USA). Alternatively, the foreign gene could produce an enzyme that corrects an abnormality in the disease state. For example, the phenylalanine hydroxylase gene could be used to normalize the elevated phenylalanine blood levels in a genetic mouse model of phenylketonuria.

In the liver, the hepatic vein is an efferent blood vessel since it normally carries blood away from the liver into the inferior vena cava. Also in the liver, the portal vein and hepatic arteries are afferent blood vessels in relation to the liver since they normally carry blood towards the liver. In a preferred embodiment, plasmid DNA may be efficiently expressed if delivered by a retrograde route into the efferent vessel of the liver (i.e. the hepatic vein). As demonstrated in the examples that follow, injections were directed into the inferior cava which was clamped in two locations; proximal and distal to the entry of the hepatic vein into the inferior vena cava. Specifically, the downstream inferior vena cava clamp was placed between the diaphragm and the entry point of the hepatic vein. The upstream inferior vena cava clamp was placed just upstream of the entry point of the renal veins. Since the veins of other organs such as the renal veins enter the inferior vena cava at this location, not all of the injection fluid went into the liver. In some of the animals that received retrograde injections in the inferior vena cava, the hepatic artery, mesenteric artery, and portal vein were clamped (occluded).

### C. Permeability

The efficiency of the polynucleotide delivery and expression was increased substantially by increasing the permeability of a blood vessel within the target tissue. Permeability is defined here as the propensity for macromolecules such as polynucleotides to move through vessel walls and enter the extravascular space. One measure of permeability is the rate at which macromolecules move through the vessel wall and out of the vessel. Another measure of permeability is the lack of force that resists the movement through the vessel wall and out of the vessel. Vessels contain elements that prevent macromolecules from leaving the intravascular space (internal cavity of the vessel). These elements include endothelial cells and connective material (e.g. collagen). High permeability indicates that there are fewer of these elements that can block the egress of macromolecules and that the spaces between these elements are larger and more numerous. In this context, high permeability enables a high percentage of polynucleotides being delivered to leave the intravascular space; while low permeability indicates that a low percentage of the polynucleotides will leave the intravascular space.

The permeability of a blood vessel can be increased by increasing the intravascular hydrostatic pressure. In a preferred embodiment, the intravascular hydrostatic pressure is increased by rapidly (from 10 seconds to 30 minutes) injecting a polynucleotide in solution into the blood vessel which increases the hydrostatic pressure. In another preferred embodiment, hydrostatic pressure is increased by obstructing the outflow of the injection solution from the tissue for a period of time sufficient to allow delivery of a polynucleotide. Obstructing means to block or impede the outflow of injection fluid, thereby transiently (reversibly) blocking the outflow of the blood. Furthermore, rapid injection may be combined with obstructing the outflow in yet another preferred embodiment. For example, an afferent vessel supplying an organ is rapidly injected and the efferent vessel draining the tissue is ligated transiently. The efferent vessel (also called the venous outflow or tract) draining outflow from the tissue is also partially or totally clamped

for a period of time sufficient to allow delivery of a polynucleotide. In the reverse, an efferent is injected and an afferent vessel is occluded.

In another preferred embodiment, the intravascular pressure of a blood vessel is increased by increasing the osmotic pressure within the blood vessel.

- 5 Typically, hypertonic solutions containing salts such as NaCl, sugars or polyols such as mannitol are used. Hypertonic means that the osmolality of the injection solution is greater than physiologic osmolality. Isotonic means that the osmolality of the injection solution is the same as the physiological osmolality (the tonicity or osmotic pressure of the solution is similar to that of blood). Hypertonic solutions
- 10 have increased tonicity and osmotic pressure similar to the osmotic pressure of blood and cause cells to shrink.

- The permeability of the blood vessel can also be increased by a biologically-active molecule in another preferred embodiment. A biologically-active molecule is a protein or a simple chemical such as histamine that increases the permeability of
- 15 the vessel by causing a change in function, activity, or shape of cells within the vessel wall such as the endothelial or smooth muscle cells. Typically, biologically-active molecules interact with a specific receptor or enzyme or protein within the vascular cell to change the vessel's permeability. Biologically-active molecules include vascular permeability factor (VPF) which is also known as vascular
- 20 endothelial growth factor (VEGF). Another type of biologically-active molecule can also increase permeability by changing the extracellular connective material. For example, an enzyme could digest the extracellular material and increase the number and size of the holes of the connective material.



## EXAMPLES

The following examples are intended to illustrate, but not limit, the present  
5 invention.

### Example 1

#### Intraportal injections of plasmid DNA:

##### Methods:

10

After the livers of 25 g, 6-week old mice were exposed through a ventral  
midline incision, solutions containing pBS.CMVLux plasmid DNA (described  
below) were manually injected over approximately 30 sec into the portal vein using  
a 30-gauge, 1/2 -inch needle and 1-ml syringe. In some animals, a 5 X 1 mm,  
15 Kleinert-Kutz microvessel clip (Edward Weck, Inc., Research Triangle Park, NC)  
was applied during the injection at the junction of the hepatic vein and caudal vena  
cava. Anesthesia was obtained from intramuscular injections of 1000 µg of  
ketamine-HCl (Parke-Davis, Morris Plains, NJ) in 1 ml of normal saline and  
methoxyflurane (Pitman-Moore, Mudelein, IL. USA) which was administered by  
20 inhalation as needed. was purchased from Sigma. Heparin was purchased from  
LyphoMed (Chicago, IL).

#### Reporter Genes and Assays

25 The pBS.CMVLux, plasmid DNA was used to express luciferase from the  
human immediate early cytomegalovirus (CMV) promoter (I. Danko, et al., Gene  
Therapy 1, 114 (1994) incorporated herein by reference). At two days after  
injection, the livers were assayed for luciferase expression as previously reported  
(J. A. Wolff, et al., Science 247, 1465 (1990)) except modified as below. The  
30 animals were sacrificed by cervical dislocation and the livers (average weight of 1.5  
g) were divided into six sections composed of two pieces of median lobe, two

pieces of left lateral lobe, the right lateral lobe, and the caudal lobe plus a small piece of right lateral lobe. Each of the six sections were placed separately into 200  $\mu$ l of lysis buffer (0.1% Triton X-100, 0.1M K-phosphate, 1 mM DTT pH 7.8) that was then homogenized using a homogenizer PRO 200 (PRO Scientific Inc.,  
5 Monroe CT). The homogenates were centrifuged at 4,000 rpm for 10 min. at 4 ° C and 20  $\mu$ l of the supernatant were analyzed for luciferase activity. Relative light units (RLU) were converted to pg of luciferase using standards from Analytic Luminescence Laboratories (ALL, San Diego, CA). Luciferase protein (pg) =  $5.1 \times 10^{-5} \times \text{RLU} + 3.683$  ( $r^2 = 0.992$ ). Total luciferase/liver was calculated by  
10 adding all the sections of each liver and multiplying by 23 to account for dilution effects. For each condition, the mean total luciferase/liver and the associated standard deviation are shown.

#### Results:

15

After the livers of 25 g, 6-week old mice were exposed through a ventral midline incision, 100  $\mu$ g of pBS.CMVLux, plasmid DNA in 1 ml of solutions was injected into the portal vein via a 30-gauge, 1/2 -inch needle over approximately 30 sec. Two days after injection, a mean of only 0.4 ng of total luciferase/liver was  
20 produced when the DNA was delivered intraportally in an isotonic solution without ligation of the hepatic vein (Table 1). Inclusion of 20% mannitol in the injection solution increased the mean total luciferase/liver over ten-fold to 4.8 ng (Table 1).

In order to prevent the DNA's rapid transit and to increase the intraportal hydrostatic pressure, the hepatic vein was clamped for two min after injection.  
25 Luciferase production increased another three-fold to 14.7 ng (Table 1).

When the DNA was injected in a hypertonic solution containing 0.9% saline, 15% mannitol and 2.5 units/ml of heparin to prevent microvascular thrombosis and with the hepatic vein clamped, luciferase expression increased eight-fold to 120.3 ng/liver (Table 1). These results are also shown in Table 7 (no  
30 dexamethasone condition) in Example 3 below for each individual animal. If the

mannitol was omitted under these conditions, luciferase expression was ten-fold less (Table 1).

These results indicate that hypertonicity, heparin and hepatic vein closure are required to achieve very high levels of luciferase expression.

5

Table 1. Mean total luciferase in the liver following the intraportal injection (over 30 seconds) of 100  $\mu$ g pBS.CMVLux in 1 ml of different solutions with no clamp or with the hepatic vein and inferior vena cava clamped for two minutes.

Condition	Mean Luciferase (total ng/liver)	Standard Error	Number of Livers
no clamp, normal saline solution (NSS)	0.4	0.7	n=6
no clamp, 20% mannitol	4.8	8.1	n=3
clamp, 20% mannitol	14.6	26.3	n=9
clamp, 2.5 units heparin/ml in NSS	11.8	12.5	n=4
clamp, 15% mannitol and 2.5 units heparin/ml in NSS	120.3	101.5	n=12

10

Luciferase activities in each liver were evenly distributed in six divided sections assayed (Table 2). All six parts of each liver from all three animals had substantial amounts of luciferase. This is in marked contrast to the direct interstitial, intralobar injection of DNA in which the expression is restricted to the site of injection (R. W. Malone et al., J. Biol. Chem **269**, 29903 (1994); M. A. Hickman, et al., Hum. Gene Ther. **5**, 1477 (1994) incorporated herein by reference).

15

Table 2. The distribution of luciferase expression over the six liver sections in animals injected intraportally (over 30 seconds) with 100  $\mu$ g of pBS.CMVLux in 1

ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes.

Liver Section	Total luciferase/Liver (ng/Liver/mouse)		
	Mouse #1	Mouse #2	Mouse #3
1/2 of median lobe	496.5	66.9	304.5
other 1/2 of median lobe	177.0	126.1	241.4
1/2 of left lateral lobe	763.8	208.7	325.2
other 1/2 of left lateral lobe	409.4	160.4	218.9
right lateral lobe	527.8	129.7	216.2
caudal lobe + small piece of right lateral lobe	374.1	149.7	240.8
Total	2,748.6	841.5	1,547.0
Mean	458.1	140.3	257.8
Range	177-763	67-209	216-325
Standard Deviation	194.0	46.6	45.9

#### Conclusions:

5

1. High levels of luciferase expression were obtained from injecting 100 µg of pBS.CMVLux intraportally.

2. The highest levels of luciferase expression were obtained when the animals were injected intraportally over 30 seconds with 100 µg of pBS.CMVLux in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes.

10

3. These high levels of expression were consistently obtained in dozens of mice.

4. The luciferase expression was evenly distributed throughout the liver.

5

### Example 2

The effects of other factors on expression were explored using the same methods for the intraportal injection of pBS.CMVLux.

#### Methods:

Unless otherwise specified, the intraportal injections and luciferase assays were done as in Example 1.

#### Results:

Compared to the results with 100 µg of pBS.CMVLUX, luciferase expression was not greater with 500 µg of plasmid DNA (Table 3). Luciferase expression was approximately 7-fold less if 20 µg of pBS.CMVLux DNA was injected instead of 100 µg.

Table 3. Total luciferase expression in each liver of each animal injected intraportally (over 30 sec) with 20 µg, 100 µg, or 500 µg of pBS.CMVLux in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein occluded for 2 min.

Total luciferase/Liver (ng/Liver/mouse)		
Mouse Number	100 µg pBS.CMVLux	500 µg pBS.CMVLux
1	1,023	15
2	178	82

3	108	23
4	140	340
Mean	362	115
Standard Deviation	441	153

The times for which the hepatic vein was occluded were varied from 2 min to 4 min and to 6 min. In Table 4, one can see that the time of occlusion did not have a large effect on expression.

5

Table 4. Effect of time of hepatic vein occlusion on luciferase expression in animals injected intraportally with 100  $\mu$ g of pBS.CMVLux in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml.

Mouse Number	Total luciferase/Liver (ng/Liver/mouse)		
	2 min	4 min	6 min
1	4.6	1.9	32.7
2	44.9	11.5	6.4

10 The times over which the injections were done were varied from 30 seconds to 1 minute and 2 minutes. In Table 5, one can see that injecting the 1 ml of the DNA solution (100  $\mu$ g pBS.CMVLux) over 30 seconds enabled the highest levels of luciferase expression. Longer times of injection led to lower levels.

15 Table 5. Effect of length of injection (time it took to inject all of the 1 ml) on luciferase expression in animals injected intraportally with 100  $\mu$ g of pBS.CMVLux in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein occluded for 2 min.

Mouse Number	Total luciferase/Liver (ng/Liver/mouse)		
	30 sec	1 min	2 min

1	2,697	188	21.6
2	790	13.4	19.9
3	1,496	141.1	11.8
Mean	1,662	114	18
Standard Deviation	964	91	5

If the total volume of the injection fluid was 0.5 ml instead of 1.0 ml, luciferase expression decreased 70-fold (Table 6) suggesting that 0.5 ml was not sufficient to fill the intravascular space and distribute the DNA throughout the parenchyma.

Table 6. Total luciferase expression in each liver of each animal injected intraportally (over 30 sec) with 100  $\mu$ g of pBS.CMVLux in either 0.5 or 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein occluded for 2 min.

Total luciferase/Liver (ng/Liver/mouse)		
Mouse Number	0.5 ml	1 ml
1	1.6	51.9
2	4.7	124.8
3	0.4	266.9
Mean	2.3	147.9
Standard Deviation	2.3	109.4

#### Conclusions:

- The optimal conditions are in fact the conditions first described in example 1: the animals were injected intraportally over 30 seconds with 100  $\mu$ g of

pBS.CMVLux in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes.

2. Use of 500 µg of pBS.CMVLux did not enable greater levels of expression  
5 but expression was approximately 7-fold less if 20 µg of DNA was used.

3. Occluding the hepatic vein for longer than 2 minutes did not increase expression.

10 4. Injecting the pBS.CMVLux over 30 seconds gave the highest luciferase levels as compared to injection times longer than 30 seconds.

5. Injecting the pBS.CMVLux in 1 ml gave higher luciferase levels than injecting the pBS.CMVLux in 0.5 ml.

15

### Example 3

#### Methods:

The intraportal injections and luciferase assays were performed as in Example 1 except that some animals received daily subcutaneous injections of 1  
20 mg/kg of dexamethasone (Elkins-Sinn, Cherry Hill, NJ) starting one day prior to surgery. The conditions for the injections were intraportal injections over 30 seconds with 100 µg of pBS.CMVLux in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes.

#### 25 Results:

Under the conditions described above (i.e., hypertonic solution containing heparin and hepatic vein closure) into animals that had been injected with daily injections of dexamethasone starting the day prior to plasmid injection, luciferase expression was three-fold greater than the expression without dexamethasone  
30 (Table 7).



Table 7. The effect of dexamethasone injections on luciferase expression after the intraportal injection of pBS.CMVLux.

Total luciferase/Liver (ng/Liver/mouse)		
Mouse Number	NO Dexamethasone	WITH Dexamethasone
1	51.9	1,181.1
2	124.8	364.7
3	266.9	82.8
4	73.7	120.5
5	52.6	1,022.9
6	7.3	178.1
7	146.1	107.6
8	231.4	140.2
9	271.2	
10	8.7	
11	8.3	
12	201.1	
Mean	120.3	399.8
Standard Deviation	101.4	444.1

- 5 Dexamethasone could have increased the production of luciferase and the expression of other genes by several mechanisms. They include increasing the amount of plasmid DNA that enters the liver cells by modifying the state of the liver cells. It could also help the liver cells withstand the high pressure. However, the most likely mechanism is that dexamethasone directly stimulates the CMV
- 10 promoter and thereby directly increases expression of luciferase by stimulating transcription of the luciferase messenger RNA.

The use of dexamethasone demonstrates that using a readily available pharmaceutical, the levels of expression can be substantially increased and regulated.

5   **Conclusion:**

1.     Dexamethasone administration increased luciferase expression from intraportally-injected pBS.CMVLux plasmid DNA three-fold.
- 10    2.     This demonstrates that the expression from the liver can be regulated using a commonly-used pharmaceutical.

**Example 4**

**Methods:**

- 15       The intraportal injections were performed using the previously stated technique of injections over 30 seconds with 100 µg of plasmid DNA in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes. The mice also received daily subcutaneous injections of 1 mg/kg of dexamethasone (Elkins-Sinn, Cherry Hill, NJ) starting one
- 20   day prior to surgery.

- The plasmids pBS.CMVLacZ and pBS.CMVnLacZ were used to express a cytoplasmic and nuclear β-galactosidase protein, respectively, from the CMV promoter (Picard, D. & Yamamoto, K. EMBO J. 6:3333-3340, 1987; incorporated herein by reference). They were constructed by placing either a 3.5-kb-
- 25   HindIII/XbaI B-galactosidase sequence from pSDKLacZpa (Danko, I. et al. Gene Therapy 1:114-121, 1994; incorporated herein by reference) or a sequence encoding a nuclear-localizing -galactosidase (Picard, D. & Yamamoto, K. EMBO J. 6:3333-3340, 1987; incorporated herein by reference) into pBlueCMV (Danko, I. et al. Gene Therapy 1:114-121, 1994; incorporated herein by reference).

- 30       Two days after intraportal injection, the livers were perfused with 1% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffered saline (PBS)

and then kept in this solution for one day. After the livers were stored in 30% sucrose, they were cryosectioned. The sections were mounted on slides and stained for 1 hour to one day with a PBS solution (pH 7.5) containing 400 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Sigma), 5 mM potassium ferricyanide, 5 mM ferrocyanide, and 1mM MgCl<sub>2</sub>. After washing, the sections were then counter-stained with hematoxylin and eosin. In the livers injected with the nuclear-localizing β-galactosidase vector, the washing step after hematoxylin incubation was omitted to decrease its nuclear staining.

10

### Results:

Having defined the optimal conditions, the types and percentages of transfected cells were determined. After injections of a 100 µg of the cytoplasmic (pBS.CMVLacZ) or the nuclear (pBS.CMVnLacZ) β-galactosidase expression vectors into dexamethasone-treated animals, liver cryosections 10- to 30-µm thick were stained for β-galactosidase using X-gal at pH 7.5 to prevent background staining. Intense blue staining was observed in approximately 1% of the liver cells and was evenly distributed throughout the liver. X-gal incubations for only 1 hour resulted in intensely blue cells; suggesting that the transfected cells expressed relatively large amounts of the foreign genes. Control livers injected with 100 µg of pBS.CMVLux did not contain any positively-stained cells. Necrosis was observed in approximately 10% of the sections. However, some livers with high β-galactosidase expression did not contain any sections with necrosis.

The hepatocytes were identified by their characteristic morphology. For example, many of the cells in the livers injected with the nuclear β-galactosidase vector, pBS.CMVnLacZ, had blue staining in two nuclei, which is a trait only of hepatocytes. Although the majority of the positively-stained cells were hepatocytes a few small, non-hepatocyte cells contained blue staining.

30

**Conclusion:**

1. Approximately 1 % of the liver cells were transfected with the B-galactosidase gene throughout the entire liver.

5

2. Almost all of the transfected liver cells were hepatocytes.

**Example 5**

10

**Methods:**

Luciferase expression in the liver was compared to that in cultured HepG2 hepatocytes in 35-mm plates. Transfections were done using 3  $\mu$ g of pBS.CMVLux/plate and either 3  $\mu$ g of Lipofectin (Life Technologies, Bethesda, MD) or 6  $\mu$ g of LipofectAMINE (Life Technologies, Bethesda, MD) per manufacturer's instructions. Two days after transfection, 200  $\mu$ l of lysis buffer was added to the cultures and 20  $\mu$ l of the supernatant were analyzed for luciferase activity as in Example 1.

20

**Results:**

The efficiency of luciferase expression using this technique was compared to other methods of gene transfer both *in vitro* and *in vivo*. Transfections performed under optimal conditions with pBS.CMV LUX and Lipofectin or LipofectAMINE (Life Technologies Inc.) in HepG2 hepatocytes in culture (n=8) yielded a mean total of  $3.7 \pm 4.5$  ng luciferase/35-mm plate and  $2.8 \pm 2.0$  ng luciferase/35-mm plate. Thus the efficiency of transfection (without dexamethasone) in terms of ng of luciferase/ $\mu$ g of pBS.CMV LUX DNA was approximately 1 ng/ $\mu$ g both *in vitro* and *in vivo*.

30

The published procedure of repetitively and directly injecting naked plasmid DNA into a rat liver lobe was reduced proportionately for mouse liver (R. W. Malone et al., *J. Biol. Chem* **269**, 29903 (1994); M. A. Hickman, et al., *Hum. Gene Ther.* **5**, 1477 (1994); incorporated herein by reference). A total of 100 µg of pBS.CMV LUX in a total volume of 200 µl of normal saline was injected within five different sites (40 µl/site) into the left lateral lobe of 30 g mice treated with dexamethasone. A mean total of only 0.1 ng/liver (4 livers; 0.001 ng luciferase/µg DNA) was obtained and the luciferase expression was only present in the injected lobe. Approximately 30-fold more luciferase expression was obtained if the direct intralobar injections were done using 1 ml of injection fluid and clamping the hepatic vein. In the previous studies involving the multiple injections of a total of 500 µg of pCMVL into a liver lobe of dexamethasone-treated rats, a mean of 9.87 ng of luciferase/liver (0.02 ng/µg DNA) was expressed (R. W. Malone et al., *J. Biol. Chem* **269**, 29903 (1994); M. A. Hickman, et al., *Hum. Gene Ther.* **5**, 1477 (1994)).

With regard to muscle, we typically inject 10 µg of pBS.CMV LUX or pBS.RSV LUX ((Danko, I. et al. *Gene Therapy* 1:114-121, 1994)) in normal saline into 6-8 mouse quadriceps muscle per experiment. In dozens of experiments, mean total luciferase per muscle was 0.4-1 ng ( $\pm 0.5-1.2$ ) and the efficiency was 0.04-0.1 ng luciferase/µg DNA.

Table 8. Comparison of efficiency of gene transfer in terms of luciferase expressed per µg of pBS.CMV Lux plasmid DNA used for the method (ng luciferase/µg DNA).

Method of Gene Transfer	Mean Total Yield Of Luciferase (ng)	Amount of pBS.CMV Lux Used (µg )	Efficiency (ng Luciferase/µg DNA)
Intraportal Mouse Liver (above optimal conditions- Table 1) hepatic vein clamped	120.3 $\pm$ 101.5 n=12	100	1.2

HepG2 In Vitro with Lipofectin	3.7±4.5 (n=8)	3	1.2
HepG2 In Vitro with LipofectAMINE	2.8±2.0 (n=8)	3	0.9
Intralobar Mouse Liver (20 ul/site X 5 sites) hepatic vein not clamped	0.1±0.1 n=4	100	0.001
Intralobar Mouse Liver (1 ml/1 site) hepatic vein clamped	2.8±5.6 n=4	100	0.028
Intralobar Rat Liver (5 sites) from published data (Journal of Biologic Chemistry 269:29903, 1994; Human Gene Therapy 5:1477, 1994)	9.87	500	0.02
Intramuscular	0.4-1 ±0.5-1.2 n>50	10	0.04-0.1

**Conclusions:**

1. The intraportal delivery of naked DNA was more than an order of  
5 magnitude more efficient than interstitial delivery into either liver or muscle and  
more evenly distributed.

**Example 6****10 Methods:**

The intraportal injections were done using the above optimal injections  
which are intraportal injections over 30 seconds with 100 µg of pCMVGH in 1 ml

of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes. Some animals received daily intramuscular injections of 100 mg/kg of cyclosporine (Sandimmune, Sandoz) or daily subcutaneous injections of 1 mg/kg of dexamethasone (Elkins-Sinn, Cherry Hill, NJ), or both starting one day prior to surgery.

The previously described pCMVGH plasmid DNA was used to express human growth hormone (hGH) (C. Andree, et al., Proc. Natl. Acad. Sci. U.S.A. 91, 12188 (1994); incorporated herein by reference). Blood obtained from the retro-orbit sinus was analyzed for serum concentration of hGH using the radioimmune assay (RIA), HGH-TGES 100T kit from Nichols Institute (San Juan Capistrano, CA).

#### Results:

Human growth hormone (hGH) was used as a marker gene to assess the ability of this gene transfer technique to produce a therapeutic serum protein (Table 9). Two days after the intraportal injection of 100 µg of pCMVGH under the above optimal conditions, the mean hGH serum concentration was  $57 \pm 22$  ng/ml ( $n=12$ ) with a range of 21-95 ng/ml. Neither dexamethasone nor cyclosporine pre-treatment significantly affected these initial hGH levels. In two animals injected with pBS.CMVLUX, background hGH levels were  $0.3 \pm 0.1$  ng/ml for 4 weeks afterwards.

In humans, normal pulsatile levels of GH peak at approximately 20 ng/ml above baseline values of approximately 1 ng/ml and can attain concentrations of 10-180 ng/ml after growth hormone releasing hormone (GHRH) stimulation (A. Favia, J. D. Veldhuis, M. O. Thorner, M. L. Vance, J. Clin. Endocrinol. Metab. 68, 535 (1989); R. W. Holl, M. L. Hartman, J. D. Veldhuis, W. M. Taylor, M. O. Thorner, J. Clin. Endocrinol. Metab. 72, 854 (1991); W. S. Evans et al., Am. J. Physiol. 252, E549 (1987); F. P. Alford, H. W. G. Baker, H. G. Burger, J. Clin. Endocrinol. Metab. 37, 515 (1973); incorporated herein by reference). The half-life of hGH is approximately 20 min in humans and 4.5 min in mice; hence these serum levels could translate into much higher levels for more stable proteins (S.

Peeters and H. G. Friesen, *Endocrinol.* **101**, 1164 (1977); A. Favia, J. D. Veldhuis, M. O. Thorner, M. L. Vance, *J. Clin. Endocrinol. Metab.* **68**, 535 (1989); incorporated herein by reference). For example, if a protein such as alpha-antitrypsin has a half-life that is ten times longer than human GH, then the circulating blood levels should be at more than ten times higher given the same efficiency of protein production. Another example is that for hemophilia which requires levels of factor VIII or IX in the range of approximately 1  $\mu\text{g}$  of the clotting factor/ml of blood. Given the increased stability of these clotting factors, then the 0.1  $\mu\text{g}$  /ml of hGH that we can achieve after intraportal injection of the respective gene means that we would be able to obtain therapeutic levels of clotting factors to prevent bleeding in patients with hemophilia. In summary, these results demonstrate that the intraportal naked DNA technique could be used to produce therapeutic levels of a circulating blood protein.

Serial measurements of hGH serum levels enabled the stability of expression in individual mice to be assessed (Table 9). In untreated animals, hGH expression was unstable as in previous studies in which the plasmid DNA was delivered to non-hepatectomized livers using polylysine complexes or intralobar injections of naked DNA.

An immune response could kill hepatocytes expressing the human protein. To test the hypothesis that expression was unstable because of an immune response, hGH levels were followed in animals that received cyclosporine with or without dexamethasone administration (Table 9). After an acute drop off, hGH levels remained at 6-11 ng/ml for four weeks in animals that received both dexamethasone and cyclosporine. In animals that received dexamethasone alone or cyclosporine alone, hGH expression was prolonged as compared to the non-treated animals but not to the same extent as the animals that received both agents. The ability for this gene transfer method to enable expression of a foreign gene should increase its utility.

Table 9. Mean serum levels (ng/ml of serum) of human growth hormone (hGH) following intraportal administration of pCMVGH under optimal conditions in mice



(2 to 3 animals for each timepoint) receiving various treatments. Optimal conditions are defined as the use of 0.9% saline, 15% mannitol, 2.5 units/ml heparin solution that was intraportally injected with the hepatic vein closed.

DAYS AFTER INJECTION	NONE	CSA alone	DEX alone	CSA + DEX
2	69	43	72	51
4	11	8	14	14
8	3	6	7	13
12	0	4	7	15
15	0	3	5	13
21	0	1.5	2.6	9.7
28	0	1	2.2	7.9

## 5 Conclusions:

1. These results demonstrate that the intraportal naked DNA technique could be used to produce therapeutic levels of a circulating blood protein that is currently used to treat humans.

10

2. The levels of the circulating blood protein (i.e. hGH) remained elevated for at least one month after a single injection.

## Example 7

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### Methods:

After the portal veins of 25 g, 6-week old mice were exposed through a ventral midline incision, 100 µg of pBS.CMVLux plasmid DNA in 0.5 ml or 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml were manually injected over 30 seconds into the portal vein near the junction of the

20

splenic vein and portal vein. The portal vein had two clamps placed distal and proximal to the point of injection so as to direct the injection fluid into only the splenic vein and to prevent the injection fluid from going to the liver or intestines.

The injections were done using a 30-gauge, 1/2 -inch needle and 1-ml syringe. 5 X

- 5 1 mm, Kleinert-Kutz microvessel clips (Edward Weck, Inc., Research Triangle Park, NC) were used. Anesthesia was obtained from intramuscular injections of 1000 µg of ketamine-HCl (Parke-Davis, Morris Plains, NJ) and methoxyflurane (Pitman-Moore, Mudelein, IL. USA) which was administered by inhalation as needed. was purchased from Sigma. Heparin was purchased from LyphoMed  
10 (Chicago, IL).

Two days after injection the spleens and pancreas were removed and placed in 500 ul of lysis buffer and 20 ul were analyzed for luciferase expression as described above.

#### 15 **Results:**

Substantial amounts of luciferase activity were obtained in the spleen and pancreas of all four mice with both injection fluids of 0.5 ml and 1 ml.

Table 10. Luciferase expression after the intravascular-administration of  
20 pBS.CMVLux into the splenic vein via the portal vein.

Total luciferase/Organ (pg/organ/mouse)		
Injection Volume	Spleen	Pancreas
0.5 ml	814.4	97.2
0.5 ml	237.3	88.7
1 ml	168.7	109.4
1 ml	395.0	97.7
Mean	403.9	98.3
Standard Deviation	289.6	8.5

**Conclusion:**

1. Intravascularly-administered plasmid DNA can express efficiently in spleen  
5 and pancreas.

**Example 8****10 Methods:**

100 µg of pBS.CMVLux in 10 ml of normal saline solution plus 15% mannitol was injected into the femoral artery of adult rats with the femoral vein clamped. One to four days after injection, the quadricep was removed and cut into  
15 10 equal sections. Each sections were placed into 500 ul of lysis buffer and 20 ul were assayed for luciferase activity as described above.

**Results:**

- 20 Substantial amounts of luciferase expression were expressed in the quadriceps following the intravascular delivery of plasmid DNA.

Table 11. Luciferase expression in the quadricep of a rat after the injection of 100 µg of pBS.CMVLux into the femoral artery and with the femoral vein clamped.

Rat Number	Total Luciferase (pg/quadriceps)
1	157.5
2	108.8
3	139.2
4	111.3

Mean	129.2
Standard Deviation	23.4

**Conclusion:**

- 5 1. Intravascularly-administered plasmid DNA can express efficiently in muscle.

**Example 9**

10 The previous examples involved injections into the afferent blood vessels of organs. In the liver, the hepatic vein is an efferent blood vessel since it normally carries blood away from the liver into the inferior vena cava. Also in the liver, the portal vein and hepatic arteries are afferent blood vessels in relation to the liver since they normally carry blood towards the liver.

15 These set of experiments were designed to determine whether plasmid DNA could be efficiently expressed if delivered by a retrograde route into the efferent vessel of the liver (i.e. the hepatic vein).

Since another luciferase expression vector was used, pCILuc, the results obtained with the hepatic vein injections were directly compared to results using  
20 the above technique of injecting the portal vein.

**Methods:**

100 µg of pCILuc in 1 ml of normal saline solution plus 15% mannitol and  
25 2.5 units heparin/ml were injected over 30 seconds into hepatic vein via the inferior vena cava. Since it was difficult to directly inject the hepatic vein in rodents, the injections were directed into the inferior cava which was clamped in two locations; proximal and distal (i.e. downstream and upstream) to the entry of the hepatic vein

into the inferior vena cava. Specifically, the downstream inferior vena cava clamp was placed between the diaphragm and the entry point of the hepatic vein. The upstream inferior vena cava clamp was placed just downstream of the entry point of the renal veins. Therefore, the 1 ml of the injection fluid entered the hepatic vein and the liver. Since the veins of other organs such as the renal veins enter the inferior vena cava at this location, not all of the 1 ml of injection fluid goes into the liver.

In some of the animals that received retrograde injections in the inferior vena cava, the hepatic artery, mesenteric artery, and portal vein were clamped (occluded) for approximately five minutes immediately before and then after the injections. Specifically, the order of placing the clamps were as follows: first on hepatic artery, then portal vein, then downstream vena cava, and then upstream vena cava. It took about three minutes to place all these clamps and then the injections were done. The clamps were left in place for an additional two minutes from the time that the last clamp (upstream vena cava clamp) was placed.

The intraportal injections were performed as stated using optimal intraportal injections over 30 seconds with 100  $\mu$ g of pCILuc in 1 ml of normal saline solution plus 15% mannitol and 2.5 units heparin/ml and with the hepatic vein clamped for 2 minutes.

Some of the mice also received daily subcutaneous injections of 1 mg/kg of dexamethasone (Elkins-Sinn, Cherry Hill, NJ) starting one day prior to surgery.

The pCILuc plasmid expresses a cytoplasmic luciferase from the CMV promoter. It was constructed by inserting the cytoplasmic luciferase cDNA into the pCI (Promega Corp., Madison, WI) CMV expression vector. Specifically, a NheI/EcoRI restriction digestion fragment containing the cytoplasmic luciferase cDNA was obtained from pSPLuc (Promega Corp.) and inserted into pCI plasmid DNA that was digested with NheI and EcoRI, using conventional recombinant DNA techniques (Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) in Molecular Cloning Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Two days after the injections, the luciferase activity was measured as above in six liver sections composed of two pieces of median lobe, two pieces of left

lateral lobe, the right lateral lobe, and the caudal lobe plus a small piece of right lateral lobe.

**Results:**

- A. Inferior Vena Cava/Hepatic Vein Injections with the Portal Vein and Hepatic Artery Clamped** (\*Injections in animal #3 were not optimal since the fluid leaked during the injections.) Injections were done in 6-week old animals that received dexamethasone.

Sections	Luciferase Activity (ng)		
	Animal #1	Animal #2	Animal #3*
1	5,576.7	4,326.4	1,527.4
2	8,511.4	4,604.2	1,531.6
3	5,991.3	5,566.1	2,121.5
4	6,530.4	9,349.8	1,806.3
5	8,977.2	4,260.1	484.2
6	9,668.6	6,100.2	1,139.3
total liver	45,255.5	34,206.9	8,610.4
mean	29,357.6		
standard deviation	18,797.7		

- B. Inferior Vena Cava/Hepatic Vein Injections with the Portal Vein and Hepatic Artery not Clamped.** Injections were done in 6-week old animals that did not receive dexamethasone.

Sections	Luciferase Activity (ng)	
	Animal #1	Animal #2
1	360.6	506.2
2	413.5	724.7
3	463.0	626.0
4	515.5	758.6

5	351.6	664.8
6	437.8	749.6
<hr/>		
total liver	2,542.0	4,029.8

mean	3,285.9
standard deviation	1,052.1

C. Portal Vein Injections with the Hepatic Vein Clamped in 6 month old mice that received dexamethasone.

Luciferase Activity (ng)			
Sections	Animal #1	Animal #2	Animal #3
1	287.4	417.0	129.2
2	633.7	808.1	220.5
3	689.8	1,096.5	328.2
4	957.8	1,056.9	181.6
5	660.7	1,487.4	178.6
6	812.4	1,276.4	233.4
<hr/>			
total liver	4,041.8	6,142.2	1,271.5

mean	3,818.5
standard deviation	2,443.0

**D. Portal Vein Injections with the Hepatic Vein Clamped in 6 week old mice that received dexamethasone.**

Sections	Luciferase Activity (ng)		
	Animal #1	Animal #2	Animal #3
1	352.9	379.1	87.0
2	667.5	373.9	108.2
3	424.8	1,277.9	178.4
4	496.3	1,308.6	111.9
5	375.2	296.4	162.3
6	434.7	628.7	123.0
total liver	2,751.4	4,264.7	770.9
mean	2,595.7		
standard deviation	1,752.1		

5 **E. Summary Table Comparing the Luciferase Expression Obtained Using the Above Conditions.**

Injection Condition@	Mean Total Luciferase/Liver ( $\mu\text{g/liver}$ )	Times Condition D.
Condition A	29.4	11.3 X
Condition B	3.3	1.27 X
Condition C	3.8	1.46 X
Condition D	2.6	1.00 X

Condition A= Inferior Vena Cava/Hepatic Vein Injections with the Portal Vein and Hepatic Artery Clamped in 6 week-old animals that received dexamethasone.

- 10 Condition B= Inferior Vena Cava/Hepatic Vein Injections with the Portal Vein and Hepatic Artery not Clamped in 6-week old animals that did not receive dexamethasone.



Condition C= Portal Vein Injections with the Hepatic Vein Clamped in 24 week old mice that received dexamethasone.

Condition D= Portal Vein Injections with the Hepatic Vein Clamped in 6 week old mice that received dexamethasone.

5

**Conclusions:**

1. Retrograde delivery of plasmid DNA into the efferent vessels of the liver via the hepatic vein/inferior vena cava leads to high levels of gene expression.

10

2. The highest levels were achieved using this retrograde approach if the afferent vessels to the liver (portal vein and hepatic artery) were occluded.

3. The CILuc plasmid enabled much higher levels of luciferase expression than the pBS.CMVLux plasmid (see above examples) using the portal vein approach in both 6-week old and 6-month old mice.

15

4. Under all conditions, luciferase expression was evenly distributed throughout all six liver sections.

20

**Example 10**

Animals that received injections into the inferior vena cava were assayed for luciferase to determine whether retrograde delivery into the efferent vessels (veins) of other organs enable gene expression.

25

**Methods:**

In the same animals that were injected using condition A above (Inferior Vena Cava/Hepatic Vein Injections with the Portal Vein and Hepatic Artery Clamped in 6 week-old animals that received dexamethasone), the kidneys were removed and assayed for luciferase as described above.

30

In the same animals that were injected under condition B above (Inferior Vena Cava/Hepatic Vein Injections with the Portal Vein and Hepatic Artery NOT Clamped in 6-week old animals that did not receive dexamethasone), the adrenal gland and diaphragm muscle, abdominal muscles, and back muscles were removed  
 5 for luciferase analysis.

### Results.

#### A. Luciferase Activity in Kidneys in Animals Injected Under Condition A.

Total Luciferase Activity/Kidney (pg/kidney)			
	Animal #1	Animal #2	Animal #3*
Right Kidney	10,827.8	7,662.3	636.3
Left Kidney	733.1	753.8	479.7

\* Injection fluid leaked.

#### 10 B. Luciferase Activity in Adrenals and Various Muscles Injected Under Condition B.

Total Luciferase Activity/Tissue (pg/tissue)			
	Animal #7	Animal #8	Animal #9
right adrenal	not assayed	82.0	49.9
left adrenal	not assayed	48.4	42.2
diaphragm	41.9	67.9	117.6
abdomen	40.4	43.9	44.0
back	37.7	40.1	40.9

15

### Conclusions:

1. Retrograde delivery of plasmid DNA into the efferent vessels of several different tissues led to substantial levels of foreign gene expression in the tissues.

2. These tissues include the adrenal glands (suprarenal glands), the diaphragm muscle, back muscles and abdominal muscles.
  3. Foreign gene expression in the diaphragm would be especially useful for
- 5 Duchennes muscular dystrophy since humans with this disorder die from respiratory failure due to fibrosis of the diaphragm muscle

### Example 11

This example explores the use of more accessible vessels such as the

10 hepatic vein and the bile duct, for delivering the naked pDNA in mice. Efficient gene expression was obtained using these efferent delivery routes. Occlusion of other vessels to restrict outflow of the injection solutions enhanced but was not critical for efficient expression. Repetitive injections into the bile duct were also accomplished. Preliminary results are also presented in larger animals, the rat and

15 dog. The incorporation of these findings into laboratory and clinical protocols is discussed.

#### Materials and Methods:

Plasmid Constructs: The pCILuc plasmid expresses a cytoplasmic luciferase from the

20 human CMV immediately early (hCMV IE) promoter. It was constructed by inserting the luc<sup>+</sup> gene, an Nhe I- EcoR I luc<sup>+</sup> fragment from pSLuc<sup>+</sup> (Promega, Madison, WI), into the pCI expression vector (Promega). pCILux expresses peroxisomal luciferase under control of the hCMV IE promoter. It was constructed by inserting the luciferase gene (Hind III - Bam HI fragment from pBlueCMVLux) into the Sma I site

25 of pCI. pCILacZ was constructed by placing the E. coli LacZ gene (Pst I - Apa I fragment pBS-RSV-LacZ) into the pCI vector (Sma I site). The pCMVGH construct was previously described (Andree, et al., 1994).

Injection Methods: Plasmid delivery into the hepatic vessels was performed in 6-week

30 old ICR mice, 2.5-6.25 month-old, 200-300 gram Sprague-Dawley rats, and beagle dogs. Ventral midline incisions were performed to expose the liver and the associated vessels. The mice were anesthetized with intramuscular injections of 1000 µg of

ketamine-HCl (Parke-Davis, Morris Plains, NJ) and methoxyflurane (Pitman-Moore, Mundelein, IL) which was administered by inhalation as needed. The rats were anesthetized with ether and the dogs were anesthetized with halothane by inhalation. The pDNA was injected in solutions containing 2.5 units/ml or heparin (Lypho-Med, Inc., Chicago, IL) (Qian et al., 1991) and either normal saline (0.9% NaCl) or 15% mannitol in normal saline (Sigma Chemical Co., St. Louis, MO). All animals received humane care in compliance with institutional (IACUC) guidelines.

In mice, the intraportal injections were performed as previously described (Budker, et al., 1996). 100 µg of pDNA in 1 ml were manually injected over ~30 sec using a 30-gauge, 1/2-inch needle and 1-ml syringe without occluding the portal vein upstream from the point of injection. In some animals, a 5 X 1 mm, Kleinert-Kutz microvessel clip (Edward Week, Inc., Research Triangle Park, NC) was applied during the injection at the junction of the hepatic vein and caudal vent cava.

DNA was delivered in mice to the hepatic vein via an occluded IVC. Clamps (6 X 1 mm, Kleinert-Kutz curved microvessel clip (Edward Week, Inc., Research Triangle Park, NC) were applied downstream (toward the heart) of the hepatic vein and upstream (towards the legs) of the hepatic and renal veins. Injections were done upstream of the hepatic vein. In some of the injections, the portal vein and hepatic artery were clamped using 6 X 1 mm, Kleinert-Kutz curved microvessel clips. The IVC mouse injections were also performed with 100 µg of pDNA in 1 ml that were manually injected over ~30 sec with a 30-gauge, 1/2-inch needle and 1-ml syringe.

The bile duct injections in mice were performed using manual injections with a 30-gauge, 1/2-inch needle and 1-ml syringe. A 5 X 1 mm, Kleinert-Kutz microvessel clip was used to occlude the bile duct downstream from the point of injection in order to prevent flow to the duodenum and away from the liver. The gallbladder inlet was not occluded. In some of the bile duct injections, the junction of the hepatic vein and caudal vena cava clamped as above. In yet other injections, the portal vein and hepatic artery were clamped in addition to the occlusion of the hepatic vein.

- In mice, repetitive injections into the bile duct were done by placing a polyethylene tube (I.D. 0.28 mm, O.D. 0.61 mm; Intramedic Clay Adams Brand, Becton Dickinson Co., Sparks, MD, USA) catheter into the bile duct after making a hole with a 27 gauge needle. The tubing was secured by a suture around the bile duct and tubing; thereby
- 5 occluding the bite duct. The other end of the tubing was placed outside the skin of the animal's back so that surgery was not required for repeat injections. No blood vessel occlusions were done for these repetitive administrations. After completion of the studies, anatomical examination indicated that the catheter remained in the bile duct.
- 10 In rats, the intraportal, IVC, and bile duct injections were done as in mice but with the following modifications. The injections were done through a 25-g butterfly needle using a peristaltic pump (Preston varistaltic power pump, Manostat Corp., New York, NY) over 1 or 3 minutes. The downstream IVC clamps in the IVC injections were
- 15 done downstream of the kidneys. For the portal vein injections, the portal vein and hepatic artery were clamped. The outflow through the hepatic vein was restricted in some animals by clamping the upstream and downstream IVC. In some animals the livers were first flushed with normal saline prior to DNA injection. Rat bile duct injections were done the same as mice. The rat doesn't have a gallbladder.
- 20 In some of the rat portal vein injections, a 25 G needle connected to a pressure gauge (Gilson Medical Electronics, Model ICT-11 Unigraph), was inserted into the liver parenchyma to determine the peak pressure within the liver during the injections. The statistical relationship between pressure and luciferase was done using Spearman's rank correlation. A smoothing spline regression model describing the relationship between
- 25 log luciferase and pressure was estimated using generalized additive model methodology (Hastie, 1992). Akaike's information criteria (Akaike, 1973) indicated that a smoothing spline with 2 degrees of freedom resulted in the best fit over all integer degrees of freedom between 0 and 5.
- 30 The injections in dogs were done as in rats except that an 18 gauge, 2 inch angiocath (Becton Dickinson, San Jose, CA) was used. All dogs except dog #1 were females. Table I indicates the injection conditions. For the bile duct injections, a suture was applied to occlude transiently the bile duct downstream from the point of injection. A

DeBakey multipurpose vascular clamp was applied to the cystic duct during injection to prevent the injectate from entering the gallbladder. In dogs, the DNA was pCILux.

Protein Assays: The luciferase assays were done as previously reported (Wolff, et al., 1990). One day after pCILuc injections, the animals were sacrificed and the rodent livers were divided into 6 sections composed of right lateral lobe, caudate lobe, two pieces of median lobe and two pieces of left lateral lobe. For each of the six pieces, 0.7 ml of lysis buffer (0.1% Triton X-100, 0.1M potassium phosphate, 1 mM DTF pH 7.8) was used for mice and four ml of lysis buffer were used for rat liver. For the dog livers, approximately 10% of each lobe was divided into five to 20 pieces and placed into two ml of lysis buffer. The samples were homogenized using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT) and centrifuged at 4,000 rpm for 10 min. at 4°C. Twenty µl of the supernatant was analyzed for luciferase activity. Relative light units (RLU) were converted to pg of luciferase using standards from Analytic Luminescence Laboratories (ALL, San Diego, CA). Luciferase protein (pg) =  $5.1 \times 10^{-5} \times \text{RLU} + 3.683$  ( $r^2 = 0.992$ ).

Ten-µm thick tissue sections were stained for β-galactosidase expression as previously described using 1-4 hour X-GAL incubations (Budker, et al., 1996). Hematoxylin was used for the counterstain but the alkaline step was omitted so that the hematoxylin stain remained red. The percent of blue-stained cells in the liver sections was determined by counting ~3000 cells in three sections and averaging.

Blood obtained from the retro-orbit sinus was analyzed for serum concentration of hGH using the RIA, HGH-TGES 100T kit from Nichols Institute (San Juan Capistrano, CA). Serum ALT and GGT levels were done using EKTACHEM DT slides and a KODAK EKTACHEM DT 60 ANALYZER as recommended by the manufacturer (Kodak, Rochester, NY).

Mice Luciferase Experiments: Our previous studies used the pBS.CMVLux plasmid for evaluating the optimal conditions for naked pDNA expression following portal injection. These optimal conditions were intraportal injections of 100 µg of pDNA in 1

ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. The injections were done over 30 seconds with the hepatic vein and IVC occluded. In this study, 100 µg of pCILuc injected under similar conditions yielded a mean total luciferase protein/liver of 3.73 µg/liver (Fig. 1A, PV+CL), approximately 30- times greater than that obtained with pBS.CMVLux. Part of this increase could be attributed to greater operator experience with these injection techniques. Injections with pCILuc under these conditions without clamping the hepatic vein yielded approximately 750-fold less luciferase (Fig. 1A, PV-CL).

10 The hepatic veins (via the IVC) of another set of mice were injected with 100 µg of pCILuc in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. A mean total luciferase protein/liver of 17.34 µg/liver (Fig. 1A, IVC+CL) was obtained when the portal vein was clamped as compared to a mean total luciferase protein/liver of 2.83 µg/liver (Fig. 1 A, IVC-CL) without occluding the portal vein.

15 Similar results were also obtained when bile ducts were injected with 100 µg of pCILuc in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. A mean total luciferase protein/liver of 15.39 µg/liver (Fig. 1A, BD+CL) was obtained when the hepatic vein was clamped as compared to a mean total luciferase protein/liver of 1.33 µg/liver (Fig. 1A, BD-CL) without occluding the hepatic vein. If mannitol was omitted then the bile duct injections without clamping any blood vessels yielded approximately 15-fold less luciferase ( $0.086 \mu\text{g/liver} \pm 0.06$ ,  $n = 25$ ). Clamping the hepatic artery, and portal vein in addition to the hepatic vein did not improve expression beyond what was obtained when only the hepatic vein was clamped (data not shown).

25 Serum ALT and GGT assays were performed on mice one and eight days after each of the above injections with pCILuc (4 mice for each condition). No increases in GOT were observed after any of the injections including the bile duct injections. Serum ALT levels increased to 200-400 U/L one day after portal vein and bile duct injections. 30 One day after IVC injections serum ALT levels increased to ~1500 U/L in half of the mice but was only ~250 U/L in the other half. By eight days after injection, serum ALT levels decreased to baseline levels in all animals. For positive control purposes, a

non-lethal intraperitoneal injection of 40  $\mu$ l of 50%, carbon tetrachloride in mineral oil was performed. An average of 25,900 U/L (n=4) was observed one day after injection.

- 5 Rat and Dog Luciferase Experiments: Similar injections into the portal vein, IVC (to the hepatic vein), and bile duct were done in rats (Fig. 1B). For the portal vein injections, the injection volumes were increased 15-times over that used in mice because the rat livers are ~15-times larger than mouse livers. One day after 750  $\mu$ g of pCILuc in 15 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution
- 10 were injected into the portal vein while occluding the hepatic vein, an average of 53.5  $\mu$ g of luciferase/liver was obtained (Fig. 1B, PV+CL). The efficiency of gene transfer in the rat was compared to that in mice two ways. In terms of efficiency as defined by the mg of luciferase per mg of tissue weight, the levels of expression for portal injections were 3.6  $\mu$ g of luciferase/g of tissue in rats as compared to 3.7  $\mu$ g of
- 15 luciferase/g of tissue in mice. Alternatively, in terms of efficiency as defined by the ng of luciferase per  $\mu$ g of pDNA delivered, the efficiencies of expression for portal injections were 71 ng of luciferase/ $\mu$ g DNA in rats as compared to 37 ng of luciferase/ $\mu$ g DNA in mice.
- 20 Less but still substantial luciferase expression was obtained when the injections of pCILuc were done into the efferent vessels of rats such as the IVC or bile duct (Fig. 1B). Injections of 750  $\mu$ g of pCILuc in 15 ml into the hepatic vein (via the IVC) while occluding the portal vein yielded an average of 1.5  $\mu$ g of luciferase/liver. Injections of 750  $\mu$ g of pCILuc in 5 to 8 ml into the bile duct without any outflow obstruction
- 25 yielded an average of 1.3  $\mu$ g of luciferase/liver.

- Parenchymal pressures of 12-50 mm Hg were measured in 23 rat livers during the injection of 750  $\mu$ g pCILuc in 15 or 20 ml into the portal vein while occluding the IVC (Fig. 2). Spearman's rank correlation between pressure and luciferase expression was
- 30 0.76 (two-side p-value < 0.001) indicating that pressure and luciferase were significantly positively associated. It appeared that pressures of over 40 mm Hg did not result in increased expression. The necessity of the non-linear component over and



above a simple linear fit was verified by an approximate full versus reduced F-test (p-value = 0.014), indicating that the observed plateau effect is real. Examination of both the residual quantile-quantile and the residual versus prediction plots further reveals that there are no serious violations of the regression model assumptions and therefore the regression models and p-values are valid (Fisher and van Belle, 1993).

Preliminary experiments explored the ability of naked pCILux (not pCILuc) to be expressed in dogs (Table I). In mice, in the case of intraportal injection, pCILux provided 3x lower expression than pCILuc (data not shown). In five dogs, various amounts of DNA were injected into either the bile duct with or without blocking outflow by occluding the IVC. In one animal, the DNA was injected into the IVC without any outflow blockage. All the dogs survived the procedure except animals that had the IVC occluded recovered more slowly post-operatively. The animals were sacrificed one day after the injections and dozens of tissue samples from each liver lobe were analyzed for luciferase. The luciferase expression was evenly distributed over all the lobes in each liver except in one lobe of one dog. Routine histological analysis in dog #5 (Table I) indicated that the tissue architecture was substantially disrupted suggesting that the injection volumes were too large.

Decreasing the volume of injection in dog #6 to 200 ml resulted in the best expression (Table I).

Mice and Rat  $\beta$ -Galactosidase Results: The  $\beta$ -galactosidase expression vector was used to determine the percent and type of cells that were transfected (Fig 3). As previously noted for portal vein injections (Budker, et al., 1996), the vast majority of the blue-stained cells appeared to be hepatocytes on morphological grounds but a few appeared to be endothelial or other types of cells. A preponderance of hepatocytes were also stained blue after the bile duct or IVC injections in mice or rats (Fig. 3).

Under the various injection conditions, the percent of cells paralleled the levels of luciferase expression (Figs. 1 and 3). In mice, the IVC injections gave the highest percentage of  $\beta$ -galactosidase-positive cells in which 7% of hepatocytes were positive

(Fig. 3B). In rats, the portal vein injections gave the highest percentage of  $\beta$ -galactosidase-positive cells in which 7% of hepatocytes were positive (Fig. 3C). In some animals, liver cell damage was evident in less than 5% of the cells (for example in Fig. 3A). Of note in the rat livers injected into the portal vein, almost all of the positively-stained cells were periacinar with few positive cells around the central vein (Fig. 3C).

Repeat Bile Duct Injections: The bile ducts of mice were cannulated and 100  $\mu$ g of pCMVhGH in 1 ml of 15% mannitol in normal saline were injected once a week (Fig. 4). Serum levels of hGH increased one day after the first injection and then decreased to background levels by seven days after injection. One day after the second injection, hGH levels again increased and then were back to background levels by seven days after the second injection. Only minimal increases in hGH levels occurred after the third injection. Mice that had the highest levels after the first injection had the lowest levels after the second injection (mice 3 and 6) and vice versa (mice 1, 2, and 4). In another set of animals (4 mice), the bile duct injections were repeated four times with pCMVhGH and then pCILuc was injected. The first three pCMVhGH injections led to similar increases in hGH serum levels as in Fig. 4. Although there were only minimal raises in hGH serum levels following the fourth injection, injection of pCILuc yielded an average of 29.2 ng/liver ( $\pm 7.1$ ,  $n=3$ ). The liver in one of the four mice was grossly yellow and scarred as a result of the bile duct ligation and did not express any luciferase.

## Discussion

This report extends the findings of the previous study showing pDNA expression following afferent intraportal delivery and demonstrates efficient plasmid expression following delivery via efferent vessels such as the hepatic vein or bile duct. Expression of luciferase or  $\beta$ -galactosidase was evenly distributed throughout the entire liver when either of the three vessels were injected. Combining these surgical approaches with improved plasmid vectors enabled uncommonly high levels of foreign gene expression in which over 15  $\mu$ g of luciferase protein/liver was produced in mice and over 50  $\mu$ g in rats (Fig. 1 and 2). Equally high levels of  $\beta$ -galactosidase expression were obtained in

that 7% of the hepatocytes had intense blue staining with only a one hour X-GAL incubation (Fig. 2). These levels of foreign gene expression are among the highest levels obtained with nonvital vectors and approach what can be achieved with vital vectors.

5

Using the portal vein administration route, occlusion of the outflow is critical for expression. Outflow occlusion increases the expression with the efferent administrative routes, but substantial amounts of expression were obtained even when the hepatic vein was not blocked. Most likely the natural direction of blood flow provides a sufficient impetus to retard the egress of injection fluid and raise the hydrostatic pressure. The use of these efferent vessels simplifies the administration for potential human applications since they are easier to access by non-invasive methods. If no occlusion is used then only one vessel has to be reached. These efferent routes should also be considered for the administration of viral and non-viral vectors as has been done with the delivery of adenoviral vectors into the bile duct [Vrancken Peeters, et al., 1996b; Vrancken Peeters, et al., 1996a; Yang et al., 1993].

15

The mechanism of pDNA uptake is not known but may involve native cellular uptake processes (Budker, et al., 1996). It is of interest that high levels of luciferase expression could occasionally be obtained when the DNA was injected into the bile duct in small volumes of isotonic solutions without occluding the IVC. Increased osmolar and hydrostatic pressure may not be critical for uptake of the pDNA by hepatocytes as they are not in muscle cells (Wolff, et al., 1990; Wolff, et al., 1991; Wolff, et al., 1992a). This would suggest that the mechanism of pDNA uptake may in fact involve endogenous cellular pathways. Increased hydrostatic and osmotic pressures may raise expression by enhancing these cellular internalization processes (Haussinger, 1996). For example, hepatocyte shrinkage stimulates replication of the duck hepatitis B virus (Offensperger, et al., 1994).

25

The raised pressures could also increase the delivery of the pDNA to the hepatocyte surface not only for the blood vessel administrations (through the sinusoidal fenestrae) but for the bile duct injections as well. The increased pressures could transiently attenuate bile secretion thereby decreasing the clearance of the pDNA (Stieger, et al.,

30

1994). For example, the hyperosmolar mannitol should induce cell shrinkage that is known to inhibit taurocholate excretion into bile (Haussinger, et al., 1992).

Hepatocytes are functionally polarized cells in which the basal and apical membranes have different exocytic, endocytic and transcytotic functions (Hubbard, et al., 1994). The success with either blood vessel or bile duct routes could indicate that both the basal (sinusoidal) and apical (bile canalicular) membranes share a common pathway for the cellular entry of pDNA. However, the passage of DNA between the basal-lateral and bile canalicular spaces cannot be excluded even under the more gentle injection conditions. Plasmid DNA injected into the bile duct could be taken up from the basal-lateral surface of hepatocytes after passage pancellularly. If only the basal-lateral surface takes up the pDNA then the increased hydrostatic and osmotic pressures may enhance pDNA expression by disrupting the tight junctions between hepatocytes and thereby increase the flow of pDNA between the canalicular and basal-lateral spaces (Desmet and De Vos, 1982).

The bile duct was cannulated to determine whether repeat injections could be done. Substantial hGH levels were obtained after the first two injections (Fig. 3). The hGH levels dropped considerably by one week after each injection presumably because of an immune response to the foreign protein. The inability to detect hGH after the third or fourth injections was most likely due to a more rapid response of the immune response sensitized to hGH. The ability to subsequently obtain luciferase expression argues against an immune response against the pDNA that prevents expression. Previous studies have failed to detect anti-DNA antibodies following the administration of naked pDNA (Nabel, et al., 1992; Jiao, et al., 1992). The ability to repetitively administer naked pDNA without inducing an immune response against the vector is a distinct advantage of naked pDNA over viral and some types of non-viral vectors.

Other studies in progress in our laboratory suggest that suppression of the immune system enables more persistent expression. In post-mitotic myofibers, plasmids can persist extrachromosomally and express for at least two years; presumably because the pDNA is not being lost as result of cell division (Wolff, et al., 1992b; Herweijer, et al., 1995). Quite possibly pDNA would be lost slowly in hepatocytes which have a half-life

of up to a year in rodents and humans (Webber, et al., 1994; Leffert, et al., 1988). If so then liver-based genetic disorders such as hemophilia could be treated by injections every six months. The bile duct could be accessed repeatedly by upper gastrointestinal endoscopy. Similarly, the hepatic vein could be non-invasively accessed via peripheral or central veins. In addition, gene transfer could be delivered to newborns via the umbilical cord vessels to get them over a newborn metabolic crisis as occurs in the organic acidurias and the urea cycle defects.

Often gene transfer techniques that work in mice do not work in larger animals. Our results demonstrate that the technique works in rats that are approximately ten-fold bigger than mice. The dog results indicate that the liver of larger non-rodent mammal can express naked pDNA. Although substantial levels of luciferase activity were obtained, further optimization of the injection conditions is required to increase the efficiency of expression so that they are comparable to those in rodents. The studies to determine the relationship between intraparenchymal pressure and luciferase expression in rats are a first step towards this goal. Minimal liver cell damage occurred in the rodents as evident by serum chemistries and histology but the injections were more disruptive to the hepatocytes in dogs. Presumably, the key factor is the efficient delivery of the pDNA to the hepatocyte surface with minimal cellular or tissue disruption (Fig. 2).

In the research laboratory, the described techniques will enable rodents to be used just as immortalized and primary liver cell cultures are now used for gene and cellular studies of liver function. The transfer of genes into cells in culture have been a critically important tool for deciphering the function of genes and for studying the effect of expressed proteins on cellular processes. Typically, the gene under study is placed within a plasmid vector and transiently transfected into the appropriate cell in culture. Isoforms and mutant forms of the gene under study can be quickly placed into plasmid expression vectors and studied. Our findings indicate that a similar plasmid-based approach could be used to study the effects of gene function in hepatocytes *in situ*. Given that the high levels of expression are transient in this system, it would be best if these effects occurred within a few days. The use of pDNA avoids the laborious steps necessary for the production of viral vectors or generation of transgenic mice and

thereby enables many different genes and their related mutated forms to be quickly studied. It will permit are mechanism of gene expression and their effects on liver function to be expeditiously probed within the context of a complete mammalian organism.

5 **Example 12**

The intravascular delivery of naked pDNA to muscle cells also has attraction. Muscle has a high density of capillaries (Browning, 1996) and the capillaries are in close contact with the myofibers (Lee 1995). However, the endothelium in muscle capillaries is of the continuous, non-fenestrated type and  
10 has low solute permeability especially to large macromolecules(Taylor,A.E.,and D.N.Granger. Exchange of macromolecules across the microcirculation. In: Handbook of Phusiology. The Cardiovascular System. Microcirculation..Bethesda, MD: Am. Physiol.Soc., 1984, sect. 2, vol. IV, chapt. 11, p. 467) The mechanism of macromolecule transendothelial transport is poorly understood. Cell biologists have  
15 proposed that this transport occurs by transcytosis involving plasmalemmal vesicles or convective transport through transient transendothelial channels formed by the fusion of vesicles. (Michel C.C. Cardiovascular Res. 1996). Physiologists have modeled the muscle endothelium as having a large number of small pore with radii of 4 nm and a very low number of large pores with radii of 20-30 nm.(Rippe B.  
20 Physiological Rev, 1994). Although the radius of gyration of 6 kb pDNA is ~100 nm (Fishman, D.M., Biopolymers, 38, 535-552), supercoiled DNA in plectonomic form has superhelix dimensions of approximately 10 nm (Rybenkov V.V. J. Mol. Biol. 267, 299-311 1997). This implies that pDNA has a possibility to traverse microvascular walls by stringing through their large pores. We hypothesized that  
25 the rate of pDNA extravasation could be increased by enhancing fluid convection through these large pores by raising the transmural pressure difference in selective regions. This report demonstrates that intravascular pDNA injections under high pressure can in fact lead to high levels of foreign gene expression in muscles throughout the selected hindlimb of an adult rat.

30 Hydrostatic Pressure

475 ug of pCILux in normal saline solution (NSS) was injected into the femoral arteries of adult Sprague-Dawley rats while blood inflow and outflow were blocked. Injection of pCILux, a luciferase expression vector utilizing the CMV promoter (LIVER II promoter accepted for HGT), was done after both the femoral artery and vein was occluded for 10 min. Two days after pDNA injections, the luciferase activities were measured in all the muscles of the hindlimb (Fig. 5). The highest levels of luciferase expression were achieved when the pCILux was injected in 9.5 ml of normal saline within 10 sec. Injection volumes less than 9.5 ml resulted in substantially less expression (Fig. 5(a)). Injection times more than 10 sec also resulted in much less expression (Fig. 5(b)). This critical dependence on volume and speed of injection indicates that either increased hydrostatic pressure or rapid flow is required for efficient expression. Artery injections performed without occluding the femoral vein resulted in approximately 200-fold less expression ( $1.8 \pm 1.2$  ng of luciferase per all hindlimb muscles).

#### 15 Other Factors

Further studies were performed to determine the effect of ischemia on the expression of the intravascularly injected pCILux. The time of ischemia was adjusted by varying the time that both the femoral artery and vein were occluded prior to pDNA injection (Fig. 5(c)). Although the highest level of expression was obtained with 10 min of ischemia, the expression levels were only a few-fold lower at shorter or longer ischemia times. This suggests that ischemia is not a critical factor for enabling egress of the pDNA out of the intravascular space. However, the blood flow for the zero ischemia time point is disrupted for ~30 sec and this may be sufficient to affect vascular permeability. Ischemia could increase expression either by capillary recruitment and vasodilatation) or augmenting permeability (Mathieu-Costello O. Int J Microcirc 1995,15, 231-237). In addition, ischemia could possibly increase pDNA expression by affecting transcription or translation. Ischemia can be tolerated by muscle for two to three hours (Gidlof A., Int. J. Microcirc. Clin. Exp. 1987, 7,67-86). Histologic analysis of the muscle did not reveal any hemorrhage or myofiber damage.

Other factors were explored to increase the level of expression. Hypotonic (Fig. 6, condition 2) or hypertonic (Fig. 6, condition 3) injection solutions resulted in less expression. Although, the effect of the hypertonic injection solution (normal saline solution with 15% mannitol) may have been mitigated by the slower rate of  
5 injection (over 30 sec instead of 10 sec) that results from its increased viscosity. The pre-injection with collagenase resulted in a 3.5-fold increase to 1,332 ng/total muscles (Fig. 6, condition 4). The collagenase was used to disrupt the basement membrane of the capillaries and thereby increase pDNA egress. It could have also increased expression by disrupting the extracellular matrix within the muscle.  
10 Further studies are in progress to determine the optimal conditions for collagenase administration without causing hemorrhage.

#### Distribution of Foreign Gene Expression

The luciferase expression was distributed among all muscle groups in the leg (Table 1). The lower levels in the lower anterior leg and foot are probably due  
15 to their high content of tendons and small amount of muscles. The levels after intravascular injection was up to 40 times higher than the levels after intramuscular injection.

The type and percentage of the transfected cells in the muscle were determined using the  $\beta$ -galactosidase reporter system. Using the best injection  
20 condition (condition 4 in Fig. 6), approximately 10-50% of the myofibers expressed  $\beta$ -galactosidase.

These expression results provide indirect evidence that pDNA extravasation occurred. More direct evidence was obtained using fluorescently-labeled DNA injected into the femoral artery (condition 1 in Fig. 6). The labeled DNA was  
25 distributed extravascularly in all the limb muscles and surrounded most of the myofibers (data not shown).

#### Conclusion

These results demonstrate that the intraarterial delivery of pDNA to muscle can be greatly enhanced when injected rapidly, in a large volume and when all



blood vessels leading into and out of the hindlimb are occluded. These conditions presumably increase the intravascular hydrostatic pressure and thereby increase the convective outflow that transports the pDNA into contact with myofibers. The high intravascular pressure may increase the number, size and permeability of the microvascular pores (Rippe, B. Acta Physiol. Scand. 125, 453-459, 1985) (Wolf, M.B. Am. J. Physiol. 257, H2025-H2032, 1989). Preliminary studies using collagenase suggest that enzymatic disruption of the vessels basement membrane or muscle extracellular matrix may also increase the delivery of pDNA to the myofibers. Ischemia also increased expression moderately. Furthermore, it is expected that other enzymes such as hylaronidase will perform similarly to collagenase.

This study shows that the intravascular approach increases expression in muscle up to 40-fold as compared to intramuscular injection. This is the first demonstration that naked plasmid DNA can be efficiently expressed in muscles of adult animals larger than mice. It is also the first report of an intravascular non-viral gene transfer approach for muscle. Further studies in larger animals will determine the clinical relevance of this study.

#### Description of Injections

A 4 cm long abdominal midline excision was performed in 150-200 g, adult Sprague-Dawley rats anesthetized with 80 mg/mg ketamine and 40 mg/kg xylazine. Microvessel clips (Edward Weck, Inc., Research Triangle Park, NC) were placed on external iliac, caudal epigastric, internal iliac and deferent duct arteries and veins to block both outflow and inflow of the blood to the leg. A 27 g butterfly needle was inserted into the external iliac artery and the DNA solution was injected by hand.

**Table 1.** Distribution of luciferase expression among the different hindlimb muscle groups two days following intraarterial injection with 475 µg of pCILux in 9.5 ml of NSS over 10. Means and their standard deviations are shown for 6 animals.

	Injection type	Muscle group	Total Luciferase	Luciferase Conc.
			(ng)	(ng/g tissue)
5	Intravascular	Upper Leg Anterior	149.1 ± 51.2	109.2 ± 37.5
		Upper Leg Posterior	74.6 ± 36.0	43.4 ± 20.9
		Upper Leg Medial	88.7 ± 63.7	61.6 ± 44.5
		Lower Leg Posterior	114.5 ± 89.7	66.0 ± 51.7
		Lower Leg Anterior	3.0 ± 1.0	5.4 ± 1.7
		Foot	0.5 ± 0.2	4.1 ± 1.4
10	Intramuscular	Upper Leg Anterior	3.7 ± 0.9	2.8 ± 0.8
15				

The foregoing is considered as illustrative only of the principles of the invention. Further, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation shown and described. Therefore, accordingly, all suitable modifications and equivalents fall within the scope of the invention.

What is claimed is:

1. A process for delivering a polynucleotide into a parenchymal cell in a mammal, comprising: transporting the polynucleotide into a vessel communicating with the parenchymal cell of the mammal such that the polynucleotide is transfected into the parenchymal cell.
2. The process of claim 1 wherein the vessel contains a fluid.
3. The process of claim 1 wherein the polynucleotide is selected from the group consisting of RNA and DNA.
4. The process of claim 3 wherein the polynucleotide provides a code for expressing a polypeptide within the parenchymal cell.
5. The process of claim 4 wherein the polypeptide is a protein.
6. The process of claim 2 wherein the vessel is a blood vessel having a permeable wall.
7. The process of claim 6 wherein the blood vessel is from the group consisting of afferent and efferent vessels.
8. The process of claim 6 wherein the permeability of the blood vessel wall is increased to allow the polynucleotide to be delivered more efficiently.
9. The process of claim 7 wherein the blood vessel is an efferent vessel.

10. The process of claim 8 wherein the permeability is increased by a method selected from the group consisting of: increasing hydrostatic pressure on the blood vessel wall, increasing osmotic pressure on the blood vessel wall and introducing a biologically-active molecule to the blood vessel wall.
11. The process of claim 10 wherein the osmotic pressure is increased by introducing a hypertonic solution to the blood vessel.
12. A process of claim 10 wherein the hydrostatic pressure is increased by obstructing outflow from the blood vessel.
13. A process for delivering a coded polynucleotide into a parenchymal cell of a mammal for expressing a protein, comprising:
  - a. transporting the polynucleotide to a vessel containing a fluid and having a permeable wall; and,
  - b. increasing the permeability of the wall for a time sufficient to allow delivery of the polynucleotide.
14. The process of claim 13 wherein the vessel is a blood vessel having intravascular pressure and a fluid outflow.
15. The process of claim 14 wherein permeability is increased by increasing the intravascular pressure to the vessel.
16. The process of claim 15 wherein increasing the intravascular pressure is performed by a method selected from the group consisting of: increasing a hypertonic pressure and increasing a hydrostatic pressure on the vessel wall.
17. The process of claim 15 wherein the hydrostatic pressure is increased by obstructing the outflow of the fluid from the vessel.

18. The process of claim 13 wherein the permeability is increased by introducing a biologically-active molecule to the vessel.
19. The process of claim 13 wherein the polynucleotide is selected from the group consisting of RNA and DNA.
20. The process of claim 13 wherein the polynucleotide is a naked polynucleotide.

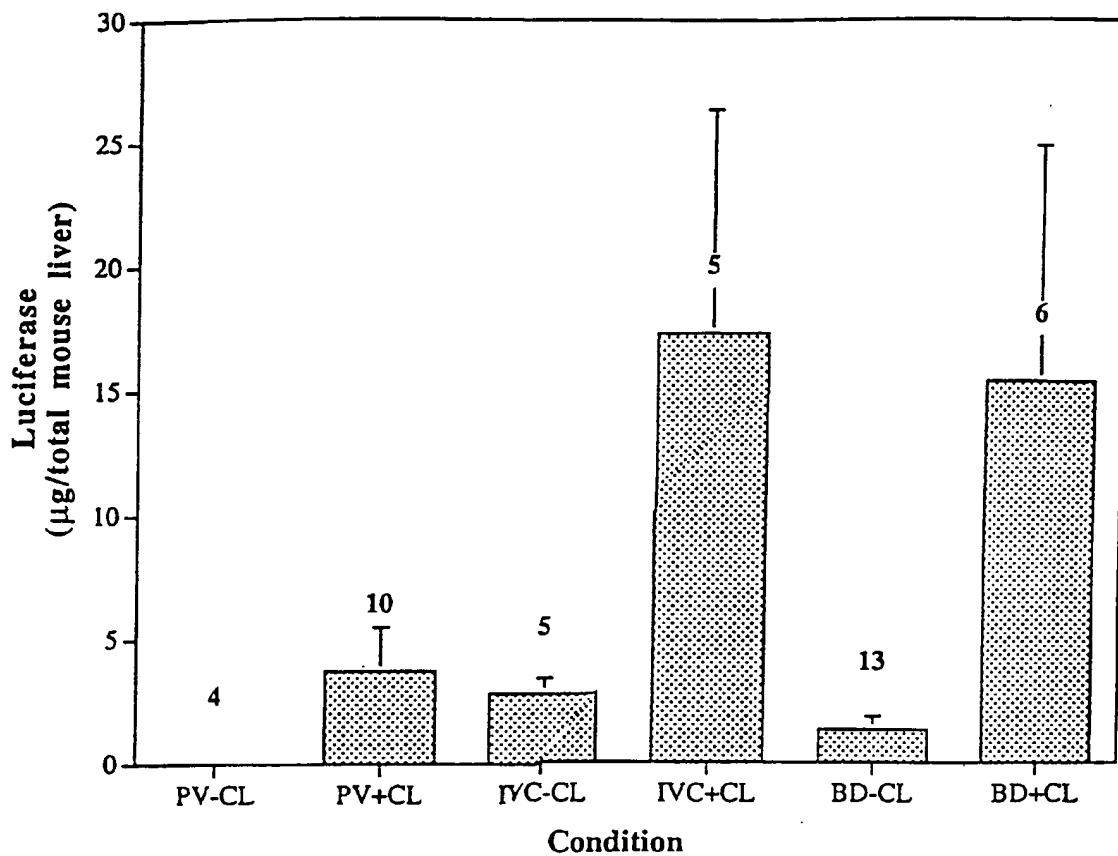


FIG. 1(a)

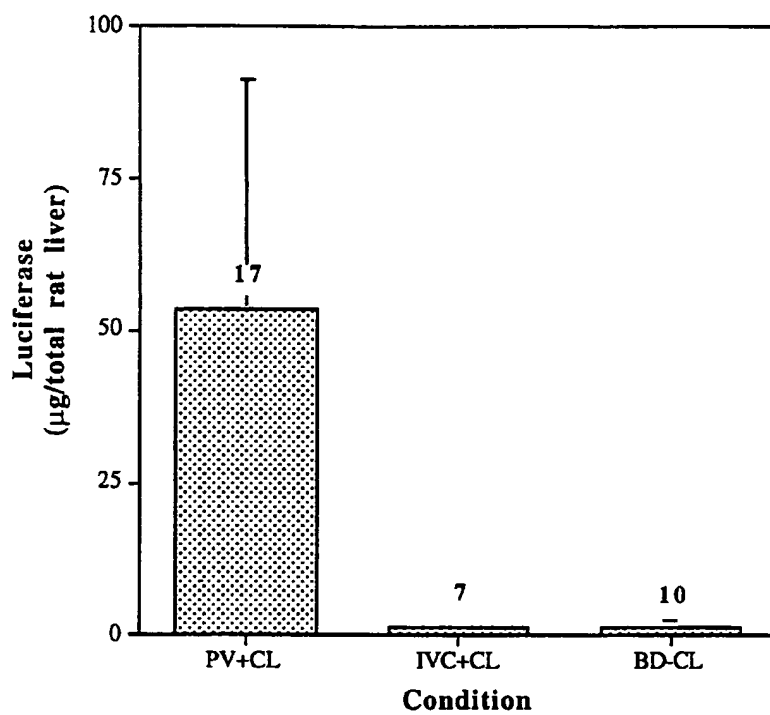


FIG. 1(b)

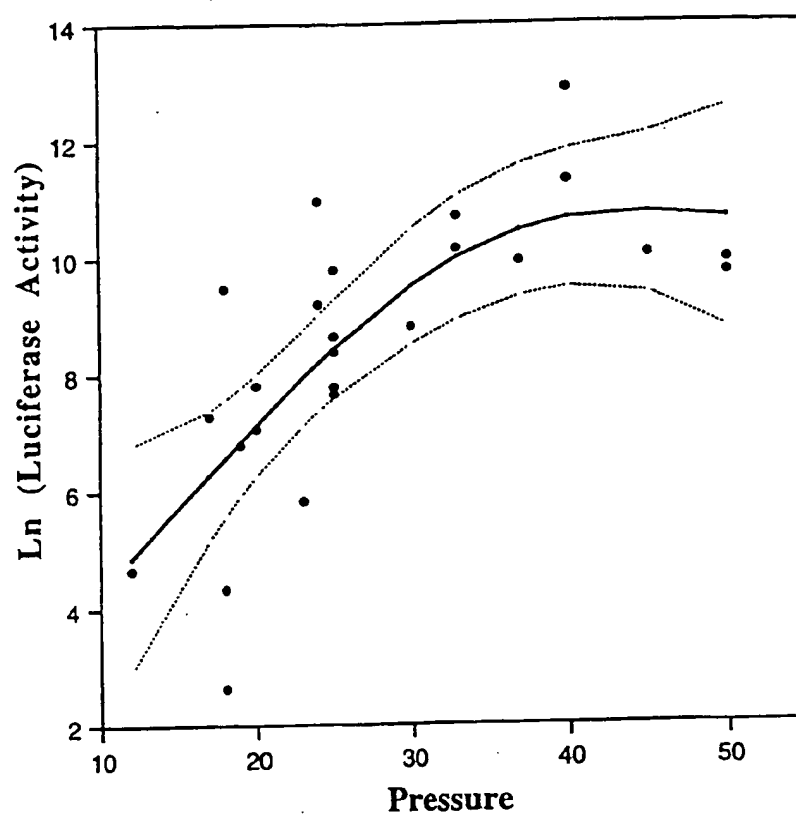
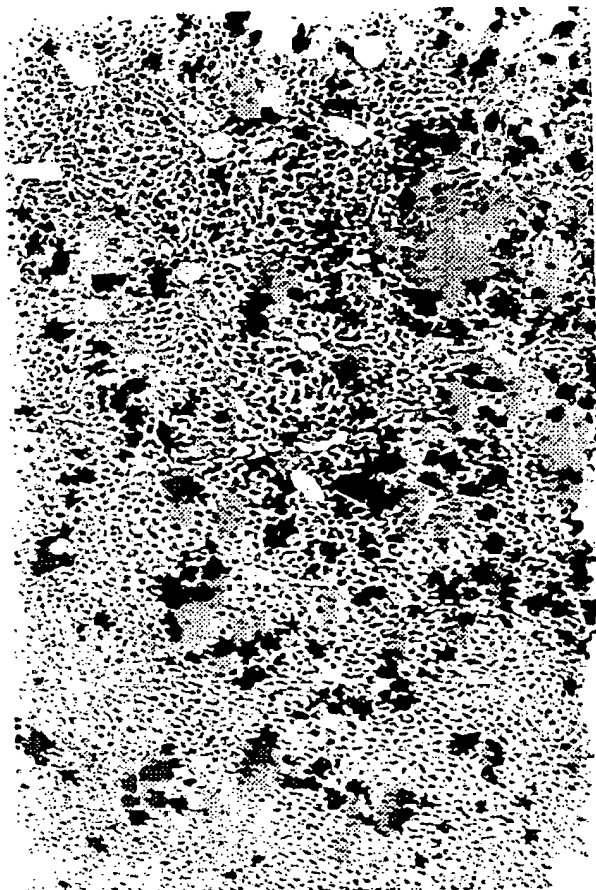
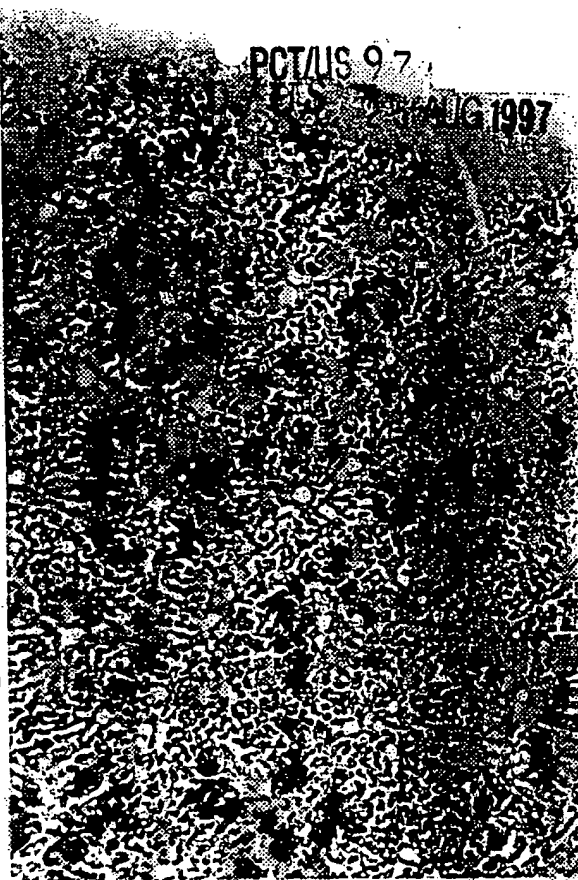


FIG. 2

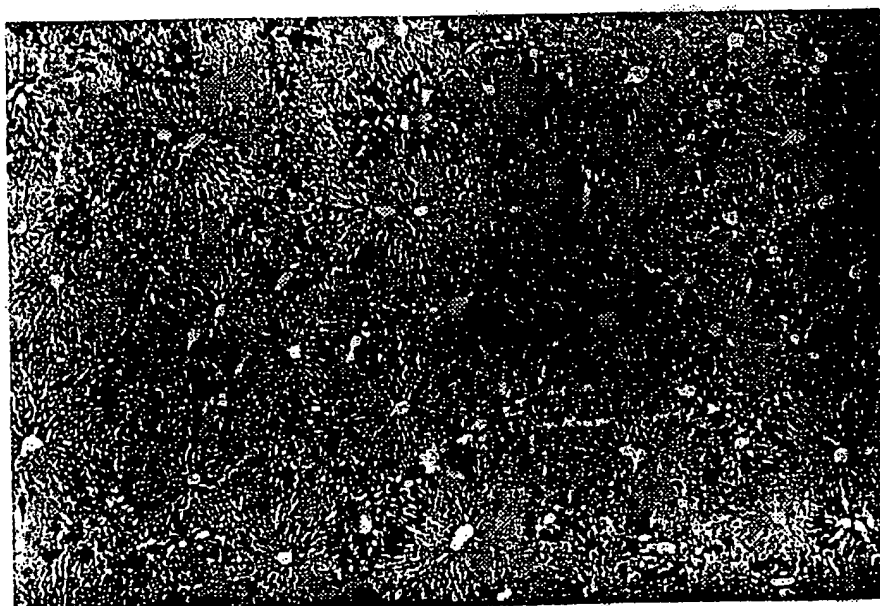




(a)



(b)



(c)

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FIG. 3(a), (b), (c)

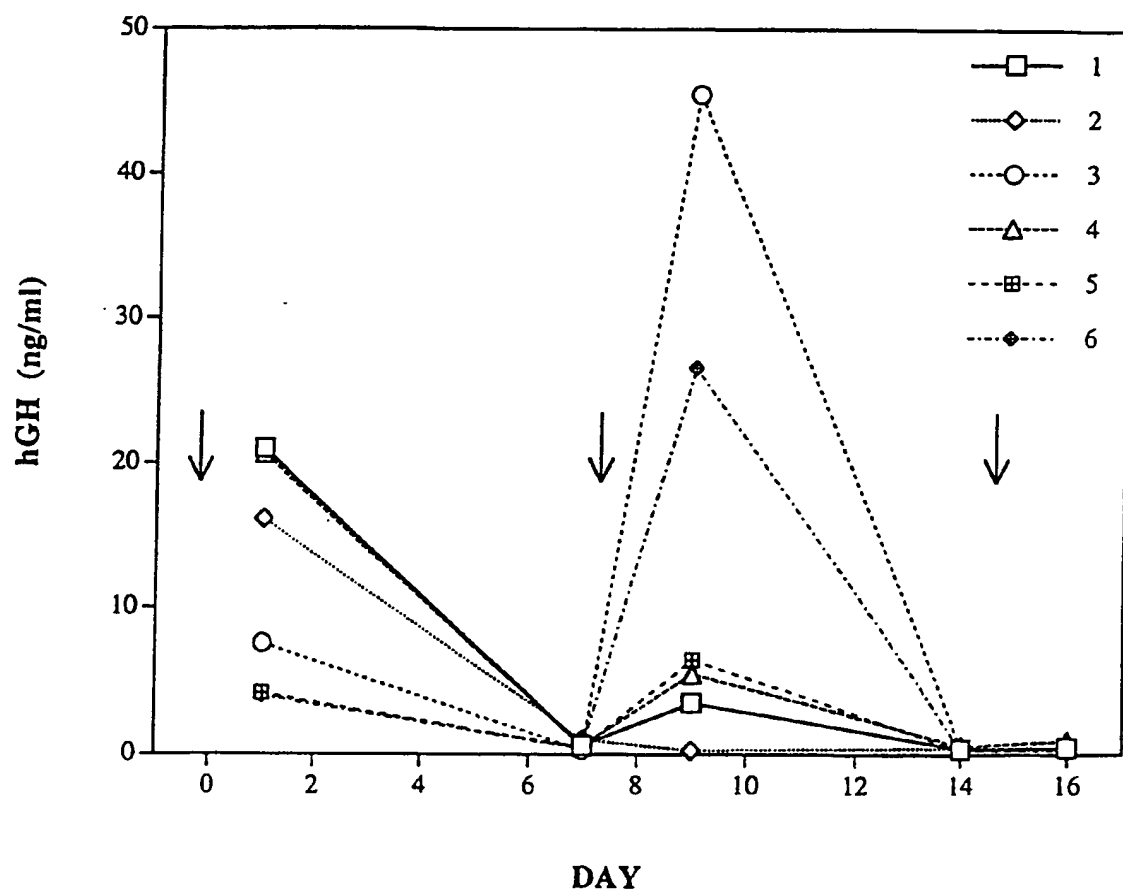
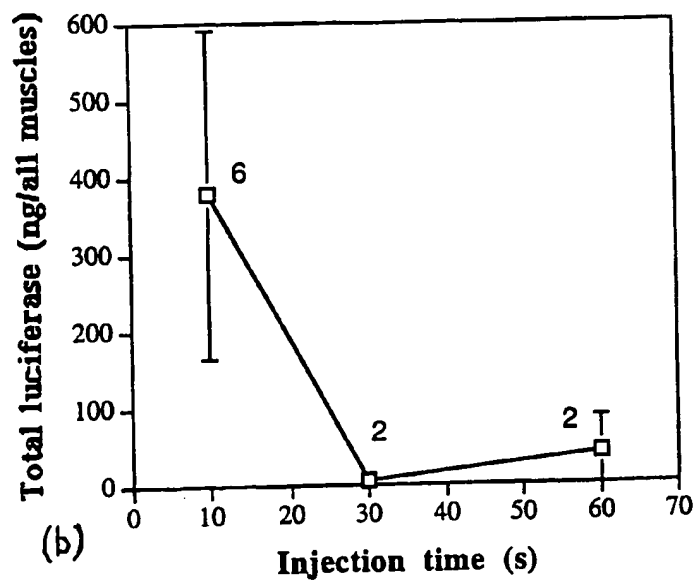
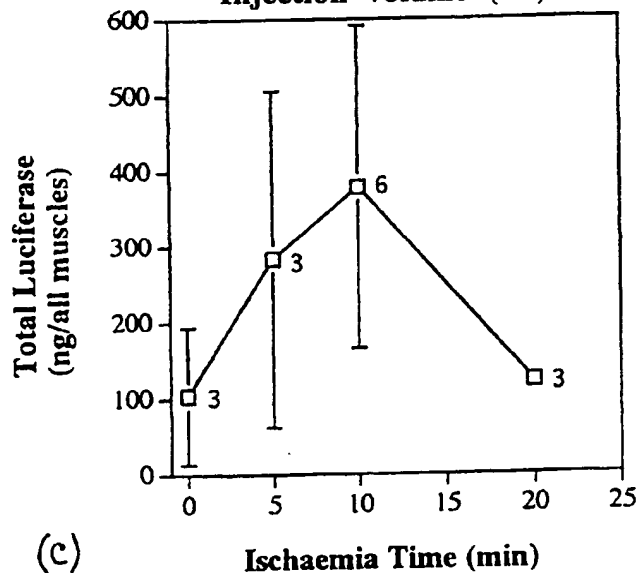
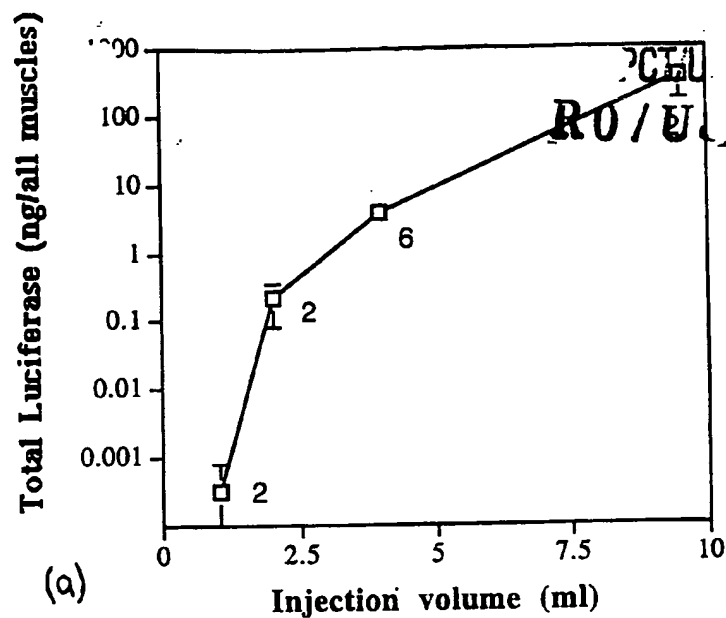


FIG. 4



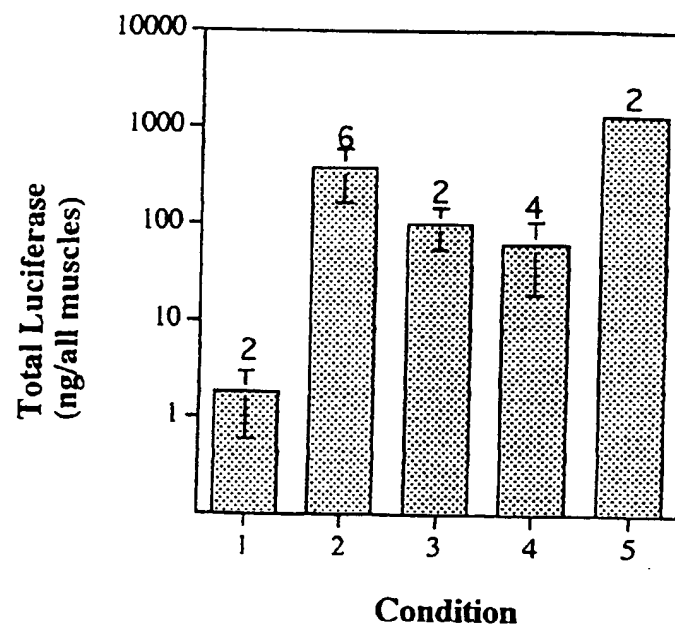


FIG. 6

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/10767

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 43/04; A61K 31/70; C12N 15/63

US CL : 514/44; 435/69.1, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/69.1, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS

search terms: vacular gene therapy, direct injection, systemic, intravenous, arterial gene transfer

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MALONE et al. Dexamethasone Enhancement of Gene Expression after Direct Hepatic DNA Injection. The Journal of Biological Chemistry. 25 November 1994, Vol. 269, No. 47, pages 29903-29907, see entire document.	1-20
Y	ZHU et al. Systemic Gene Expression After Intravenous DNA Delivery Into Adult Mice. Science. 09 July 1993, Vol. 261, pages 209-211, see entire document.	1-20
Y	NOPANITAYA et al. Effect of Hepatic Venous Outflow Obstruction on Pores and Fenestrations in Sinusoidal Endothelium. British Journal of Experimental Pathology. 1976, Vol. 57, pages 604-609, see entire document.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 08 AUGUST 1997	Date of mailing of the international search report 04 SEP 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL D. SCHMUCK Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/10767

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RIESSEN et al. Arterial Gene Transfer Using Pure DNA Applied Directly to a Hydrogel-Coated Angioplasty Balloon. Human Gene Therapy. 1993, Vol. 4, pages 749-758, see entire document.	1-20